症例 1

ペースメーカ本体露出部に感染を起こした 60 歳代日本人男性

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主訴：ペースメーカ感染，リード抜去術
家族歴：特記すべきことなし
既往歴：20 年前より糖尿病，6 年前より透析
現病歴：（X－5）年，完全房室ブロックに対しペースメーカ植込術施行．（X－8）か月前にペースメーカを交換．（X－7）か月前より，植込術創部の乖離，ペースメーカ本体の一部露出，排膿，間欠的な悪寒・発熱が出現．膿汁よりメチシリン耐性黄色ブドウ球菌（MRSA）を検出したため，linezolid（LZD，1.2g/日経口）投与を開始した．2 週間後，血小板数が減少したため，vancomycin（VCM）0.5g/透析日投与と創部の洗浄を転院日（X）まで継続した．

入院時現症：身長 170cm，体重 74kg，意識清明，体温（腋窩）37.2℃，血圧 100/50mmHg，脈拍 74/分，SpO_2（room air）98%，右上胸部：ペースメーカの露出と皮膚乖離部からの排膿あり，心雑音なし，肺音清，腹背部：特記すべき所見なし

入院時検査：WBC 8,800/μL，Hb 9.2g/dL，Plt 143,000/μL，TP 6.4g/dL，Alb 3.6g/dL，AST 21U/L，ALT 10U/L，LDH 169U/L，ALP 200U/L，T-Bil 0.4mg/dL，CPK 257U/L，BUN 25.1mg/dL，Cre 6.6mg/dL，Na 139mEq/L，K 3.8mEq/L，Cl 106mEq/L，iP 3.1mg/dL，CRP 0.85mg/dL，Hb_{A1C} 5.0%
経食道心エコー：左室機能障害（EF 27%），中等度 MR，疣贅認めず

入院後経過：（X+2）日，ペースメーカリード抜去術およびテンポラリペースメーカ挿入術施行．その後バイタルサインは安定し，VCM 投与を継続したまま（X+6）日に紹介元へ転院した．
転院後，抜去したペースメーカリードやジェネレータ周囲から採取した検体から，LZD 感性の MRSA，LZD 感性のメチシリン感性黄色ブドウ球菌（MSSA）に加え，LZD 耐性の MSSA を検出したとの報告があった[1]．

考察

1. 分離した LZD 耐性 MSSA の同定，感受性は正しいか．

当時，PubMed で文献検索したところ，LZD 耐性 MSSA を分離した事例報告はほとんどなかった[2]．
今回，分離菌の同定，薬剤感受性測定には，自動同定装置（WalkAway 96SI，Siemens Healthcare Diagnostics，Tokyo）を用いていたため，LZD 耐性 MSSA と同定された株のうちの 1 株（178-2 株）について，性状を再検討した．その結果，カタラーゼ試験およびコアグラーゼ試験（ウサギプラズマ‘栄研’，栄研化学，野木），ラテックス凝集試験（PASTOREX スタッフプラス，バイオ・ラドラボラトリーズ，東京）が陽性で，*femA* 遺伝子を保有[3]していた．また，API Staph（シスメックス・ビオメリュー，東京）での検討および 16S ribosomal RNA（rRNA）遺伝子の相同性[4]から，黄色ブドウ球菌と同定して矛盾しないと考えた．オキサシリンおよび LZD の最小発育阻止濃度（MIC）を DryPlate（栄研化学）および E-test（シスメックス・ビオメリュー）で 3 回（1 回あたり 3 セット）測定したところ，それぞれの中央値が ≤2μg/mL および 8μg/mL だったため，薬剤感受性の判定も妥当[5]と考えた．

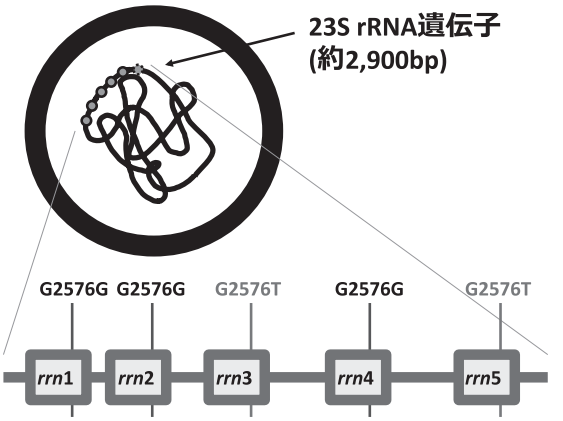
2. LZD 耐性黄色ブドウ球菌の疫学はどのようか．

2011 年に行われた米国および国際調査では，全ての MSSA が LZD 感性（MIC が 8μg/mL 未満）だった[6][7]．LZD 耐性 MRSA については，日本でも分離例の症例集積報告がある[8]ものの，LZD 耐性 MSSA の分離は極めて珍しいと考えた．

3. 178-2 株の LZD 耐性の機序はどのようか．

黄色ブドウ球菌で報告がある LZD 耐性機序には（1）23S rRNA 遺伝子の変異（2）*cfr* 遺伝子の獲得（3）L3/4 変異の 3 つがある[9]．それぞれについて 178-2 株を解析したところ，5 つの 23S rRNA 遺伝子のうち 2 つ（*rrn*3 と *rrn*5）の V 領域に，G2576T の

Fig. 1　178-2株の23S rRNA遺伝子の変異．黄色ブドウ球菌は，染色体上に5つあるいは6つの23S rRNA遺伝子を持つが，178-2株では5つの遺伝子を持っていた[10]．



塩基置換を認めた[10]（Fig. 1）．*cfr*遺伝子[11]およびL3/4変異[12)13)]は認めなかった．このため，178-2株は，23S rRNA遺伝子の変異によってLZD耐性を獲得したと考えた．23S rRNA遺伝子の変異によるLZD耐性のレベルは，変異が集積すると上昇するとの報告[8)14)]がある．178-2株では，23S rRNA遺伝子の2/5が変異しており，LZDのMICが8 μg/mLと耐性カテゴリのブレークポイント下限であることと矛盾しないと考えた．

4．178-2株はどのようにLZD耐性を獲得したか．

今回，抜去したペースメーカリードやジェネレータ周囲の検体から，LZD感性MRSA，LZD感性MSSA，LZD耐性MSSA（178-2株を含む）を複数株検出した．そのうち，保管されていたLZD感性MRSAとLZD耐性MSSAの遺伝子型をパルスフィールドゲル電気泳動で比較したところ，遺伝子パターンが類似していた．このため，178-2株とLZD感性MRSAの1株（179-4株）について，multilocus sequence型および*spa*型を調べた．その結果，どちらの株もそれぞれST239[15]および*t*137[16]と同一型だった．このため本症例では，最初にLZD感性MRSAが患部に付着し，その一部が治療中に*mecA*遺伝子の脱落と23S rRNA遺伝子の変異を起こしてLZD耐性MSSAに変化した，と推測した．ただし，前医で分離したMRSA株，およびペースメーカ抜去時に分離したLZD感性MSSA株の微生物学的検討はできていない．また，転院半年前に使われたLZDの投与期間は2週間にすぎず，長期間投与していたわけではない．

5．LZD感性MRSAがLZD耐性MSSAに変化した，という仮説の検証するために（1）179-4株をミュラーヒントン寒天培地に塗布（2）LZDのE-testストリップをのせ一晩培養（3）阻止帯ぎりぎりから菌を回収，これを14回繰り返しLZDのMIC値の変化を観察したが，大きな変化は起こらなかった．

まとめ

黄色ブドウ球菌によるペースメーカ感染は稀ではないが，本症例では，検体から複数の感受性パターンを示す黄色ブドウ球菌を検出し，その中にLZDに耐性を示すMSSAを認めた．この株は，5つの23S rRNA遺伝子のうち2つにG2576Tの塩基置換を認め，この変異がLZD耐性の原因と考えた．同時に分離したLZD感性MRSAと遺伝子型を比較したところ，極めて類似していたため，最初にLZD感性MRSAが患部に付着し，その一部が治療中にLZD耐性MSSAに変化した，と推測した．ただし，この仮説を疫学的，微生物学的に証明することはできなかった．

＜症例の疑問点から研究的考察へ＞

1．当院で黄色ブドウ球菌の分離・同定を行う場合，グラム染色，カタラーゼ試験，コアグラーゼ試験を行った後，通常菌体やコロニーの形態および生化学的性状に大きな矛盾がなければ，自動同定装置の判定を臨床側に報告している．分離菌が黄色ブドウ球菌（さらにMRSA）であると微生物学的に厳密に定義するには，どのような条件が必要か？

2．LZD耐性黄色ブドウ球菌の日本での疫学はどのようか？

3．黄色ブドウ球菌がLZD耐性を獲得するメカニズムとMIC値には，どのような関連があるか．また，最近明らかになったメカニズムはあるか？

4．臨床的に，MRSAがMSSAに，あるいはその逆に変化することはあり得るか．また，LZDの投与期間とLZD耐性菌の発生には関連があるか？

5．感性菌に変異を起こさせて耐性菌を作る場合，どのような方法を行うのが標準的か？

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“本症例の疑問点”から“研究的考察”へ

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分離株の同定と耐性の確認

●本症例からは MSSA と MRSA が分離された.
●これらの同定精度は生化学と遺伝子検査にとって支持されている.
● LZD 耐性 MSSA が分離された.
● LZD 耐性株は G2576T の変異が *rrn*3 と *rrn*5 に確認された.
○ LZD 耐性 MSSA は間違いなく存在する.

● LZD 耐性 MSSA と LZD 感性 MRSA の 8 種類の毒素遺伝子保有状況は完全に一致した.
● LZD 耐性 MSSA と LZD 感性 MRSA の PFGE はバンドが 2 本異なる近縁型であった.
● LZD 耐 性 MSSA と LZD 感 性 MRSA の MLST（ST239）と spa type（t137）は同一であった.
○ LZD 耐性 MSSA と LZD 感性 MRSA は同一株の可能性が高い.

以上の結果から

● MSSA は MRSA から mec 領域が脱落した可能性を否定できない.
● MSSA が LZD 耐性を獲得したか，MRSA が LZD 耐性を獲得後に *mec* 領域が脱落したかは現在のデータからは読み取れない.
○ MSSA と MRSA が混在している同一プレートから LZD 感性 MSSA，LZD 耐性 MSSA と LZD 感性 MRSA を分離する検査技師の力量が問われる症例であった.

1. LZD 耐性 MRSA の出現頻度は？

2011 年の LEADER surveillance[1] と ZAAPS program[2] で検出された 1,520 株と 2,551 株の MSSA から LZD 耐性株は分離されなかった．さらに MRSA については，LEADER surveillance で 1,505 株中から 3 株（0.2%）が検出されたが，ZAAPS program の 1,333 株からは検出されなかったと報告されている．また，2004 年から 2009 年の LEADER surveillance では 23,077 株の黄色ブドウ球菌中 13 株（0.05%）しか検出されていない[3]．つまり，LZD 耐性黄色ブドウ球菌の検出は極めて少なく，その中でも MSSA が LZD 耐性となる可能性はほとんどないと判断できる．しかし，全く存在しないと言う意味

Fig. 1　リボソームの形成と蛋白合成阻害薬の機序

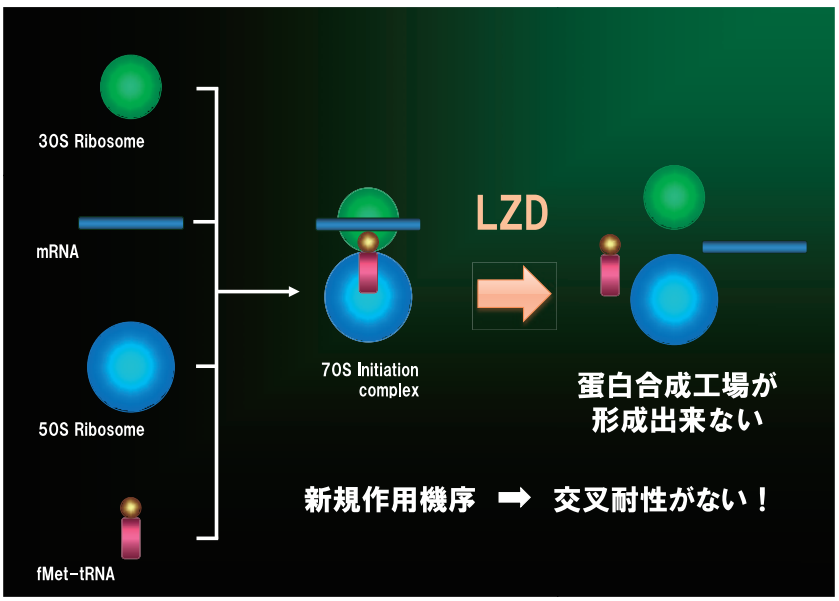
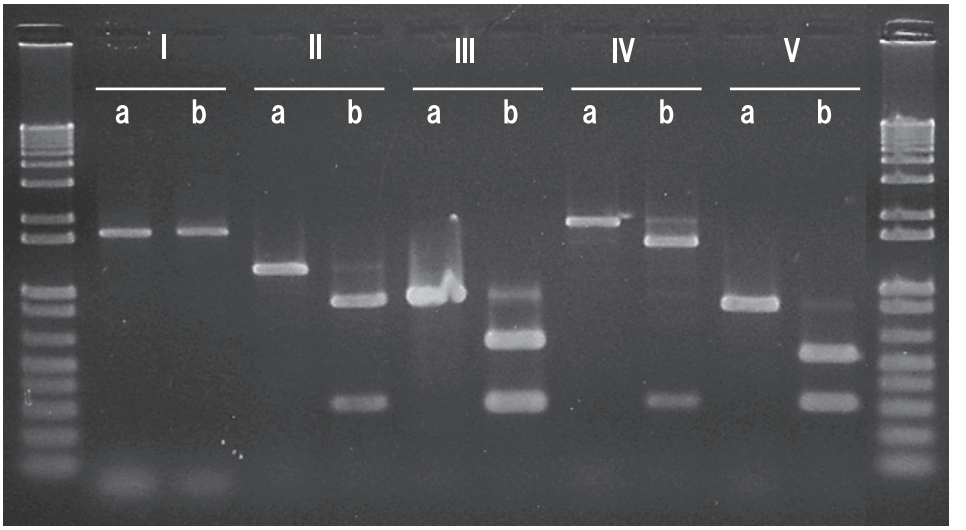


Fig. 2　Detection of LZD-resistant *S. aureus* by a simple PCR-restrict enzyme method[10)]



Gabriel EM *et al,* J Microbiol Methods. 2012 Aug;90(2):134-6

1. I, II, III, IV, V: 23S rRNAの１番～5番.
2. a: 制限酵素*Nhe*Iで処理する前
3. b: 制限酵素*Nhe*Iで３７C、３０ min 処理後

Note: 1）Primer Tm was set as 65C.
　　2）Sequencing confirmed that KUB269 has mutations in 23S rNRA alleles II to V.

ではなく，LZD 耐性 MSSA は 2012 年にスペインからの報告があり，今回の場合と同様に G2576T の変異によって LZD の MIC も 8μg/mL と同等である[4)]．

2. MSSA と MRSA は同一株か？

Pulsed-field gel electrophoresis（PFGE）の結果は，MSSA と MRSA には 1 もしくは 2 バンドの違いしかなく極めて近傍の株であった．さらに，両株とも coagulase は III 型であり，毒素は *sea*，*seb*，*sec*，*sed*，*see*，*eta*，*etb*，*tst* の順で両株とも＋，－，－，－，＋，－，－，－であった．また両株の multilocus sequence typing（MLST）は ST239 であり *spa* typing は *t*137 と同一であった．したがって上記データのみから考察すると，両株は異なった株とは考えにくく，むしろ同一株と考えた方が科学的と思われる．このような同一株か否か検証する PFGE や MLST，spa typing は長尾先生らの「MLST などによる MRSA の分子疫学」を参照されたい[5)]．

3. LZD 耐性 MSSA はどうして出現したのか？

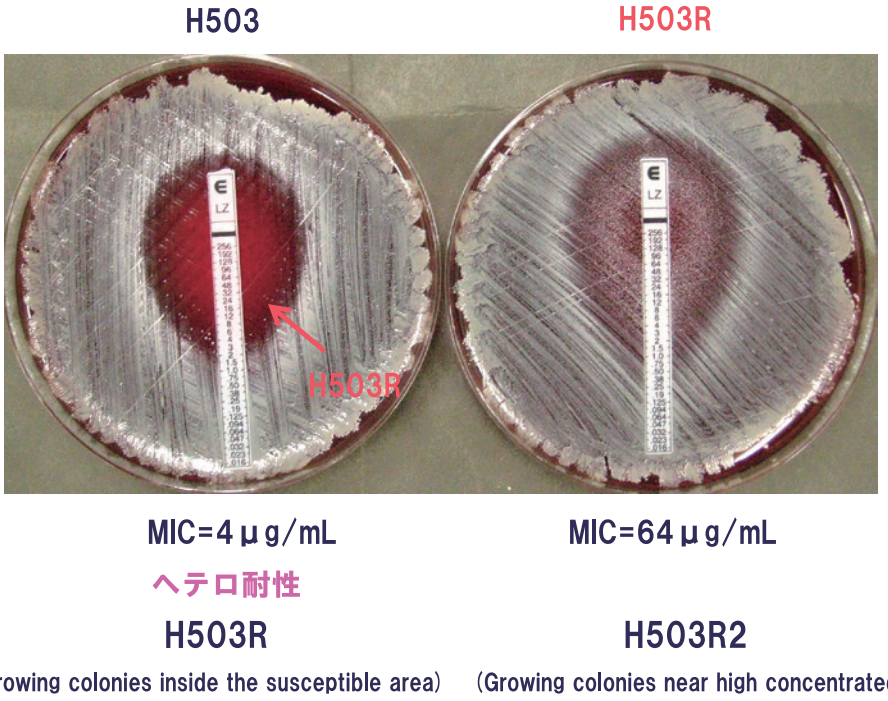
現状のデータから考察すると，同一患者から同時期に分離された MSSA と MRSA は同一株と考えられる．同一株であるなら，MRSA から SCC*mec* 領域が脱落しなければならないが，このような現象がおきるのだろうか？　人工的に VCM 耐性を獲得させると SCCmec 領域が脱落した MSSA 株が出現する事は確認されている[6)7)]．さらに，臨床現場でも MRSA の *mecA* 遺伝子を含むほとんどの SCCmec 領域が脱落し，残余する SCCmec が確認された MSSA が報告されている[8)9)]．SCCmec 領域は mobile genetic elements であり感染患者体内でも脱落がおきる可能性は充分にある．したがって，本症例で分離された MRSA の SCCmec 領域が脱落して MSSA になった可能性は大きいと考える．

さらに，なぜいつの時点で LZD 耐性になったのか？　本患者は LZD が 2 週間程度投与されているので，その期間に耐性を獲得したと考えられる．しかし，MRSA が LZD 耐性を獲得して MSSA になったのか，SCCmec 領域が脱落して MSSA となった時点で LZD 耐性を獲得したのかは不明である．特に，LZD 感性の MSSA が保存されておらず，その遺伝学背景を調査できなかった事は残念である．

4.）今後の課題

本症例は，MRSA と MSSA が同一患者から同時に分離された症例であるが，この事自体は良く見られる現象である．しかし，生化学的にも遺伝学的にも極めて近縁，ほとんど同一株と考えられる株であり，MRSA の SCCmec 領域が脱落して MSSA になったと推測できる．この事は *in vitro* でも *in vitro* でも臨床でも報告はあるのが，これらの両株が同一株か否かを検証するには全塩基配列を決定して比較すべ

Fig. 3　E-test of H503 and H503R strains on blood agar plate (48 h after inoculation)



きで今後の課題と思われる．さらに，これらの株にLZDが使われた事でLZD耐性を獲得したと考えられるが，MSSAの状態でLZD耐性を獲得するよりもMRSAの状態で獲得する方が確率的には高いと思われる．この事を臨床で証明するには詳細で継続的な検出が必要と思われる．

5.）補足

LZDの作用機序は23S rRNAのdomain Vに特異的に結合する事で蛋白合成工場自体を作らせない（Fig. 1）．したがって理論的には全ての蛋白の合成を止めてしまい，その作用機序から他の蛋白合成阻害剤との交叉耐性はない．LZDの耐性は複数の箇所の変異が報告されているが，もっとも頻度の高い変異はG2576Tであり，今回の変異もこの場所であった．この変異箇所が複数のコピーにはいると耐性度は相関的に高くなる．このコピー数は日本の株は5つあり，変異が入るとG→Tになるので特異的な制限酵素（*NheI*）で切断できる．つまり耐性遺伝子になると2本のバンドとして，感性だと1本のバンドとして確認できる方法がある（Fig. 2.）[10]．さらに耐性細胞と感性細胞が混在するとE-testではFig. 3のような現象が確認できる．一般的に耐性細胞は増殖が遅いのでLZD耐性細胞と感性細胞が混在する場合でLZDの暴露がない条件が続けば，増殖の早いLZD感性細胞が大多数を占めるようになるので，その細胞集団は感性株にかわる事が報告されている．

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ORIGINAL ARTICLE

Linezolid-resistant *Staphylococcus aureus* isolated from 2006 through 2008 at six hospitals in Japan

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Abstract Limited use of linezolid for treating methicillin-resistant *Staphylococcus aureus* (MRSA) infection was approved in Japan in 2006. We report here the status of linezolid-resistant MRSAs in Japan. Eleven linezolid-resistant clinical isolates from 11 patients at six hospitals were collected from 2006 through 2008. The minimal inhibitory concentration (MIC) of linezolid in these strains varied from 8 to 64 μg/ml. All strains had at least one G2576T mutation in the chromosomal gene(s) encoding domain V of the 23S ribosomal RNA (rRNA). Chromosomal DNA encoding five copies of the domain V region was analyzed by polymerase chain reaction (PCR). Strains with the linezolid MICs of 64, 32, 16, and 8 μg/ml had the G2576T mutation(s) in four, three (or four), two, and one copy of the 23S rRNA genes, respectively. These results suggest that the level of linezolid resistance seems to be

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roughly correlated with the number of mutations in the genes encoding 23S rRNA. DNA samples from all 11 strains were subjected to pulsed-field gel electrophoresis and were classified into seven independent clones having >92% identity. Among the 11 patients, five had been treated with linezolid and the remainder, in two hospitals, had no history of prior linezolid use. The results suggested possible nosocomial infections by linezolid-resistant MRSA.

Keywords Linezolid · Resistance · rRNA gene · MRSA · Nosocomial infection

Introduction

Infection by methicillin-resistant *Staphylococcus aureus* (MRSA) in immunocompromised patients is a serious problem in hospitals because the bacterium shows resistance to a wide variety of antimicrobial agents. More recently, MRSA infection of healthy individuals in the community has become progressively problematic. To combat MRSA infection, three types of powerful antimicrobial agent have been introduced: the glycopeptide antibiotics such as vancomycin and teicoplanin, the oxazolidinone derivative linezolid, and the aminoglycoside arbekacin.

Linezolid is the first clinically used oxazolidinone antimicrobial agent that is active against most gram-positive bacteria, including vancomycin-resistant *S. aureus* and vancomycin-resistant *Enterococcus*. Most *S. aureus*, including MRSA and the coagulase-negative *Staphylococcus*, is found to be linezolid-susceptible at the breakpoint of 4 μg/ml [1]. Linezolid shows antimicrobial activity through inhibition of protein synthesis of susceptible cells by binding to the domain V region(s) of the 23S ribosomal RNA (rRNA) and thus inactivating the function of the 50S ribosomal subunit [1, 2]. This powerful new antibiotic was first approved for clinical use in 2000 in the USA as well as European and other countries. Soon after the introduction of linezolid to clinical use, in 2001, linezolid-resistant MRSA was reported in North America [3]. To the best of our knowledge, <20 linezolid-resistant isolates from clinical specimens have been reported worldwide up to 2008 [3–7]. The most common mechanism of linezolid resistance involves a single nucleotide substitution in the chromosomal DNA encoding the domain V regions of 23S rRNA. The most frequently found mutation associated with linezolid resistance in the clinical strains of *S. aureus* is a G2576T substitution (*Escherichia coli* 23S rRNA gene numbering) [3–5, 7]. Linezolid-resistant strains generated in vitro by serial passage on a linezolid-impregnated medium confirmed the presence of the G2576T mutation [5, 8]. It was also shown in vitro that the frequency of linezolid resistance is very low, $<10^{-9}$ [8]. Another mutation found in linezolid-resistant MRSA was a T2500A substitution in the domain V region of the rRNA genes [6]. The mutation was not only limited to a single copy of the 23S rRNA gene but was found in multiple copies of the 23S rRNA genes [9]. Besides the mutation in the 23S rRNA gene(s), two additional mechanisms of linezolid resistance have been reported. One is expression of the chloramphenicol–florfenicol resistance (*cfr*) gene, which encodes 23S rRNA methyltransferase [10], and the other is a mutation in the gene(s) encoding the 50S ribosomal subunit proteins L3 and L4 designated as *rplC* and *rplD*, respectively [11, 12].

The *S. aureus* chromosome encodes rRNA at five to six independent rRNA genes (*rrn*) or operons [13]. If the G2576T mutation were to be accumulated in different copies of 23S rRNA genes in the same cell, it is conceivable that the level of resistance against linezolid would be progressively high. In fact, an in vitro study revealed that stepwise passages of linezolid-susceptible cells through medium containing progressively higher concentrations of linezolid yielded mutants with progressively high minimal inhibitory concentrations (MICs) of linezolid [5]. Analysis of such mutants showed that the cells accumulated the G2576T mutation in multiple copies of the 23S rRNA genes and the number of mutations was roughly correlated with the level of resistance [14].

The use of linezolid in Japan for treating MRSA infection was approved in 2006, 6 years later than in the USA. Emergence of linezolid-resistant MRSAs in Japan was reported (in Abstracts in the Japanese language) from three independent groups in 2008 and 2009. In addition, a case of patient who had linezolid-resistant MRSA infection was reported during the preparation of this manuscript [15]. However, a thorough analysis of linezolid-resistant strains collected from hospitals in different regions of Japan has not been reported. We report here seven independently isolated linezolid-resistant MRSAs from 11 patients at six hospitals in Japan. Nosocomial infection of the linezolid-resistant MRSA manifested in two of these hospitals.

Materials and methods

Bacterial strains

Linezolid-resistant MRSAs were collected from six different hospitals in Japan from six different regions (Table 1). Among 11 MRSA isolated, five were from patients after linezolid treatment and six were from patients who had no record of linezolid treatment. A clonal relationship of the MRSA strains was determined by pulsed-field

Table 1 Linezolid-resistant methicillin-resistant *Staphylococcus aureus* (MRSA)

Hospital	Patients	Strains[a]	Linezolid treatment (days)[b]	Specimen	Mutation	Number of mutation in the rRNA gene[c]
KY	A	KY5	29	Blood	G2576T	4
KT	B	KT1	47	Stool, blood	G2576T	2
	C	KT6	0	Sputum, stool	G2576T	At least 1
KS1	D	KS510	90	Blood	G2576T	4
KS2	E	KS227	25	Blood	G2576T	3
TH	F	TH1	0	Sputum	G2576T	At least 1
	G	TH2	0	Catheter	G2576T	At least 1
	H	TH3	0	Wound pus	G2576T	At least 1
	I	TH4	0	Wound pus	G2576T	1
	J	TH5	0	Pus	G2576T	1
TK	K	TK471	15	Blood	G2576T	At least 1

Hospital locations: *KY* (Kyushu), *KT* (Kanto), *KS1* (Kansai 1), *KS2* (Kansai 2), *TH* (Tohoku), *TK* (Tokai)

[a] A multiple strain isolated from the same patient having an identical DNA type was excluded

[b] A given number means a total number of days the patient was treated with linezolid either consecutively or intermittently

[c] All strains had five copies of the rRNA gene

gel electrophoresis (PFGE) of *Sma*-I-treated genomic DNA. Substrains repeatedly isolated from the same patient with an identical DNA type were excluded from the list.

Antibiotic susceptibility test

The MIC of the antimicrobial agent was determined by the agar dilution method according to the protocols of the Clinical and Laboratory Standards Institute (CLSI) [16].

Population study

Bacteria were grown in Mueller–Hinton broth overnight, and the cell density was adjusted to $A_{578} = 0.3$. The suspension was serially diluted tenfold, and 100-μl aliquots were streaked on linezolid-impregnated brain–heart infusion agar. Number of colonies was counted after 48 h of incubation at 35°C.

Extraction of DNA

A single colony was isolated from the overnight culture on Mueller–Hinton agar, suspended in phosphate buffer saline, and treated with 20 mg/ml of lysozyme (Sigma-Aldrich Corp., St. Louis, MO, USA) and 1 mg/ml of lysostaphin (Wako Pure Chemicals, Osaka, Japan) for 30 min at 37°C according to the manufacturer's instructions. Then the genomic DNA was extracted by the QIAGEN DNeasy Blood & Tissue kits (Qiagen GmbH, Hilden, Germany). The DNA was subjected to polymerase chain reaction (PCR) amplification.

PCR amplification of the chromosomal gene encoding the 23S rRNA

Chromosomal genes encoding the 23S rRNA of five independent operons were amplified using the primers reported earlier [9]. Sizes of the PCR product using the primers rrn1 through rrn5 were expected to be in the range of 5.6–6.5 kbp. Given these large sizes, Phusion High Fidelity DNA polymerase (Finzymes, Espoo, Finland) and GC-enriched PCR buffer were used. A thermal cycler was set as follows: 94°C for 30 s for the initial denaturation and then followed by 30 cycles of denaturation, annealing, and extension at 94°C for 20 s, 55°C for 20 s, and 72°C for 3.5 min, respectively. The PCR products were subjected to agarose-gel (1%) electrophoresis, extracted, and purified using a QIAGEN Gel extraction kit (Qiagen). PCR amplification of the domain V region of the 23S rRNA genes and DNA sequencing chromosomal DNA encoding the domain V region of the 23S rRNA spanning from the 2,280th through 2,699th bp (*E. coli* numbering) was amplified. The primers used were 5′-GCGGTCGCCTC CTAAAAG-3′ (upper primer, corresponding to the 2,280th through 2,297th bp of the *S. aureus* 23S rRNA gene) and 5′-ATCCCGGTCCTCTCGTACTA-3′ (lower primer, corresponding to the 2,680th through 2,699th bp). DNA was amplified using Phusion High Fidelity DNA Polymerase (Finzymes). PCR was carried out as follows: 98°C for 30 s, and then 35 cycles of denaturation, annealing, and extension at 98°C for 10 s, 58°C for 10 s, and 72°C for 5 s, respectively. The products were subjected to agarose-gel electrophoresis (AGE) (2%), and the gel was stained with

GelRed (Biotium, Hayward, CA, USA). The isolated DNA was sequenced (Takara Bio, Mie, Japan or Nihon Gene Research Laboratories, Miyagi, Japan) and aligned with the corresponding nucleotide sequences obtained from linezolid-susceptible *S. aureus* (GenBank accession no. X68425).

Pulsed-field gel electrophoresis

Chromosomal DNA was extracted from the MRSA cells and then digested with *Sma*-I according to the method described by Bannerman et al. [17, 18]. The DNA plugs sliced at a thickness of 1–4 mm were placed in 170 μl of a solution containing 10 U of *Sma*-I and 20 μl of T buffer [0.1 M Tris–hydrochloric acid (HCl), pH 8.0, 700 mM magnesium chloride ($MgCl_2$), 0.2 M potassium chloride (KCl), 700 mM 2-mercaptoethanol], and 20 μl of 0.1% bovine serum albumin (BSA). The mixture was incubated at 25°C for 4 h. Samples were loaded on 1% agarose gel prepared in 0.5× Tris-borate + ethylenediaminetetraacetate (EDTA) (TBE) buffer containing 44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA pH 8.0. The wells were sealed with 1% agarose in the same buffer. PFGE was carried out with a CHEF-DRIII electrophoresis cell (Bio-Rad) at 6 V/cm for 20 h at 14°C, with initial and final pulses conducted for 5.3 and 34.9 s, respectively. The gel was stained with GelRed (Biotim) according to the manufacturer's manual and visualized under a 254-nm ultraviolet light.

Results and discussion

Source of linezolid-resistant MRSA and the patient's background

We collected 11 linezolid-resistant clinical isolates from 11 patients at six hospitals through members of the MRSA Forum of Japan (Table 1). Hospitals from which the strains were collected were scattered throughout Japan: Kyushu (KY), Kansai 1 (KS1), Kansai 2 (KS2), Tokai (TK), Kanto (KT), and Tohoku (TH) hospitals. Therefore, it is unlikely that the same strain was transmitted from one hospital to another. In the KT hospital, three and two strains were isolated from patients B and C, respectively, and these are reported here as KT1 and KT6, respectively. In the KS2 hospital, two strains with slightly different properties were isolated and are reported here as KS227 due to perfect matching of the DNA type. Among the 11 patients, five had a history of linezolid treatment, with individual total treatment times of 90, 47, 29, 25, and 15 days consecutively or intermittently. Obviously, prolonged linezolid administration caused the emergence of the resistant strain in most cases. On the other hand, six strains were isolated in two hospitals from six patients who had no history of linezolid treatment, suggesting the possibility of nosocomial infections. It is of interest to note that five strains were isolated in the TH hospital from five patients who had no history of linezolid treatment, suggesting dissemination of the resistant strain in the hospital from the patient(s) transferred from other hospitals. In the KT hospital, one patient had linezolid treatment, and the identical DNA type strain was isolated from another patient who did not have linezolid treatment.

Antimicrobial susceptibility of the isolates

The MICs of linezolid varied from 8 to 64 μg/ml in these MRSA strains (Table 2). MIC values were not necessarily correlated with the duration of linezolid treatment throughout. The linezolid MIC in the strains isolated from patients who were not treated with linezolid may be unpredictable because these strains must have been transmitted from other patient(s). In fact, the linezolid MIC in six of these strains appeared to be high, at 32, 32, 16, 8, and 8 μg/ml. The strain isolated from patient C, who had no record of linezolid treatment, showed the linezolid MIC of 32 μg/ml. In the TH hospital, five strains were isolated from five patients with no history of linezolid treatment, and the linezolid MICs in these strains were 8–32 μg/ml. These results strongly indicate that the resistant MRSAs were transmitted in the same hospital or nosocomially. A substrain of KS227 isolated from patient E, who had a history of linezolid administration and subsequently vancomycin treatment, showed resistance to both linezolid and vancomycin, with MIC values of 16 and 4, respectively (data not shown). This strain may be classified as a linezolid-resistant vancomycin-intermediate-resistant MRSA.

All these strains were resistant to oxacillin, imipenem, pazufloxacin, minocycline, and chloramphenicol but were susceptible to vancomycin, teicoplanin, and arbekacin. All strains showed very high MIC values of chloramphenicol. It was reported that linezolid resistance could be conferred via chloramphenicol florfenicol methyltransferase [19, 20]. Thus, it is possible that some of these strains acquired the *cfr* gene encoding chloramphenicol–florfenicol methyltransferase. We have not investigated this possibility any further.

Classification of the linezolid-resistant MRSA strains

In order to investigate whether these linezolid-resistant MRSAs isolated in Japan were derived from different or similar clones, we analyzed the DNA type of these strains by PFGE of *Sma*-I-restricted DNA (Fig. 1). Overall relatedness of the strains isolated from different hospitals appeared to be 72%, which indicates considerable distance, suggesting that the strains isolated from different hospitals

Table 2 Minimal inhibitory concentrations (MICs) of selected antimicrobial agents commonly used for treating methicillin-resistant *Staphylococcus aureus* (MRSA) infection

Strains	LZD	CHL	VAN	TEC	ABK	SXT	RIF	MIN	PZFX	IPM	OXA
KY5	32	64	1	0.5	2	2	≦0.25	16	>128	128	>64
KT1	16	32	1	1	0.5	1	≦0.25	16	>128	32	>128
KT6	32	64	1	1	0.5	1	≦0.25	16	>128	8	128
KS510	64	64	1	1	4	2	≦0.25	16	>128	64	>128
KS227	32	32	2	2	4	2	≦0.25	16	>128	128	>128
TH1	32	64	2	4	0.5	2	≦0.25	16	16	>128	>128
TH2	16	64	1	4	1	2	≦0.25	16	16	>128	>128
TH3	8	32	1	4	0.5	2	≦0.25	16	16	>128	>128
TH4	8	32	1	4	0.5	2	≦0.25	16	16	>128	>128
TH5	8	32	1	4	0.5	2	≦0.25	16	16	>128	>128
TK471	16	64	2	2	1	2	≦0.25	8	>128	>128	>128

LZD linezolid, *CHL* chloramphenicol, *VAN* vancomycin, *TEC* teicoplanin, *ABK* arbekacin, *SXT* sulfonamide-trimethoprim, *RIF* rifampin, *MIN* minocycline, *IPM* imipenem, *OXA* oxacillin

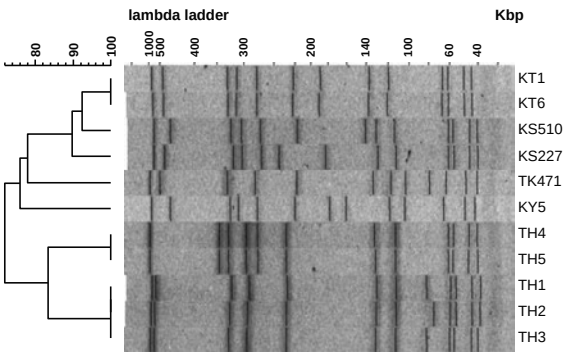

Fig. 1 Pulsed-field gel electrophoretic profiles of *Sma*-I-digested genomic DNA of the linezolid-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) and its subsequent cluster analysis

were from independent clones. Strains isolated in the KS1, KT, or KS2 hospital showed about 92% identity, suggesting close relatedness. However, it is less likely that they were derived from the same clone because of the distant localities of these hospitals—at Hyogo, Tokyo, and Osaka. The strains isolated in the TH hospital showed 83% identity; however, close examination revealed that the strains could be divided into two groups: TH1–3 and TH4–5, suggesting the presence of two independent resistant clones in this hospital. Two strains from the KT hospital showed 100% identity, indicating the dissemination of a single clone of the resistant strain within the hospital. These findings suggest the presence of nosocomial infection of the linezolid resistant strains in two hospitals. To the best of our knowledge, these were the first cases of nosocomial dissemination of linezolid-resistant MRSAs. Population analysis of linezolid MICs was carried out for all strains. The results showed that all strains tested were reasonably homogeneous, with two- to fourfold differences among the MICs (data not shown).

Mechanism(s) of linezolid resistance in the present isolates

As mentioned above, the linezolid-resistant strains used in this study were isolated from several hospitals in distant regions of Japan. Among these, more than two strains were isolated in three hospitals either from the same or different patients. Therefore, it is important to investigate the identity of the clone(s). Clonal analysis of the isolates and identification of the mutation(s) may provide important information in understanding whether the resistant strains were transmitted between hospitals or, more importantly, whether there was an occurrence of nosocomial infection. To date, the cause of linezolid resistance in *S. aureus* has been reported to be mainly a nucleotide substitution at G2576T in domain V of the rRNA genes. In addition, it is possible that the resistant strains had mutations in more than one copy of the rRNA genes. Therefore, we analyzed the chromosomal DNA encoding the rRNA of the isolates by PCR amplification and subsequent nucleotide sequencing.

It is well recognized that *S. aureus* has five or six copies of rRNA operons [9, 13]. To ascertain this, we first analyzed the chromosomal DNA encoding the rRNA gene by long-range PCR and found that all the strains had five copies of the rRNA gene. Amplification of the 23S rRNA genes of five different operons was carried out by a previously described method [9]. Needless to say, the linezolid-susceptible strain showed no sign of mutation in domain V of any 23S rRNA operons. All the linezolid-resistant isolates investigated here were subjected to analysis of domain V of the rRNA genes. The results revealed

that all isolates had at least one G2576T mutation that may have been the cause of the linezolid resistance (Table 1). To understand whether the mutation(s) in multiple copies of the rRNA gene was related to the level of resistance, we analyzed the rRNA genes of all five copies of the selected strains. The strains selected were TH4, TH5, KT1, KS227, KY5, and KS510 having the linezolid MICs of 8, 8, 16, 32, 32, and 64 μg/ml, respectively (Table 2). Result revealed that the strains with the linezolid MICs of 8, 16, 32, and 64 μg/ml had the G2576T substitution in one, two, three (or four), and four copies, respectively, of the rRNA genes. No strain having the mutation in all five operons was found, probably because such a cell may be lethal. Thus, it became evident that the accumulation of a single point mutation in different copies of rRNA genes is correlated with the level of linezolid resistance in clinically isolated MRSA.

Previous in vitro study revealed that the accumulation of a single point mutation, G2576T, in the 23S rRNA genes of different operons might be associated with a stepwise increase in the level of linezolid resistance [14]. The results of this study using clinically isolated strains were consistent with that of in vitro studies. Stability of the G2576T mutation was studied by several groups of investigators, and two contrasting results were reported. One group of investigators reported that the resistant phenotype of clinical isolates was stable even if the resistant strains were subjected to serial subculture in a linezolid-free medium [21]. Another group reported that the resistant cells subcultured for 60 cycles in a drug-free medium caused loss of mutations of the 23S rRNA gene [14, 22].

Overall, it is clear that the number of mutations in multiple copies of the 23S rRNA gene is most likely associated with the levels of linezolid resistance in the cells isolated from clinical materials, and this finding might be consistent with the result obtained from the resistant cells selected in vitro. Two groups of investigators suggested that the G2576T mutation in the ribosomal gene acts as negative selective pressure in cell growth in a linezolid-free environment because the growth rate of the mutant may be slower than that of the wild-type cells due to the low rate of protein synthesis [9, 14]. Other investigators reported that the mutation rate in the emergence of the linezolid-resistant mutant is primarily very low, but once the mutation has occurred, the rate becomes ~1,000-fold higher for unknown reasons [9]. It was also reported that some strains having the linezolid MIC of 4 μg/ml, the breakpoint, have a mutation in the 23S rRNA gene as selected in the linezolid-containing medium [14]. Surveillance studies have indicated that linezolid-resistant MRSA is still rare. However, long-term use of linezolid for MRSA infection may cause the emergence and dissemination of resistant cells. This study in fact demonstrates the nosocomial transmission of linezolid-resistant MRSA to patients who had no history of linezolid treatment.

In summary, we described the microbiological characterization of 11 clinical isolates of linezolid-resistant MRSAs in Japan. It was reported that appearance of spontaneous oxazolidinone-resistant cells is very low, $>10^{-9}$, in *S. aureus* [8], which is consistent with the fact that the frequency of linezolid-resistant clinical isolates of *S. aureus* is still low [23, 24]. However, during a short period of clinical use of linezolid in Japan for treating MRSA infection, we experienced the emergence of at least 11 (this report) plus two (meeting reports) linezolid-resistant MRSA strains.

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ORIGINAL ARTICLE

The emerging ST8 methicillin-resistant *Staphylococcus aureus* clone in the community in Japan: associated infections, genetic diversity, and comparative genomics

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Abstract Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) has become a major concern worldwide. In the United States, ST8 CA-MRSA with SCC*mec*IVa (USA300) has been predominant, affecting the entire United States. In this study, we investigated Japanese ST8 CA-MRSA with new SCC*mec*IVl (designated ST8 CA-MRSA/J), which has emerged in Japan since 2003. Regarding community spread and infections, ST8 CA-MRSA/J spread in 16.2–34.4% as a major genotype in the community in Japan, and was associated with skin and soft tissue infections (SSTIs), colitis, and invasive infections (sepsis, epidural abscesses, and necrotizing pneumonia), including influenza prodrome cases and athlete infections, similar to USA300. It spread to even public transport and Hong Kong through a Japanese family. Regarding genetic diversity, ST8 CA-MRSA/J included ST and *spa* variants and was classified into at least three pulsed-field gel electrophoresis types, ST8 Jα to γ. Of those, ST8 Jβ was associated with severe invasive infections. As for genomics, ST8 CA-MRSA/J showed high similarities to USA300, but with marked diversity in accessory genes; e.g., ST8 CA-MRSA/J possessed enhanced cytolytic peptide genes of CA-MRSA, but lacked the Panton–Valentine leukocidin phage and arginine catabolic mobile element, unlike USA300. The unique features of ST8 CA-MRSA/J included a novel mosaic SaPI (designated SaPIj50) carrying the toxic shock syndrome toxin-1 gene with high expression; the evolution included salvage (through recombination) of hospital-acquired MRSA virulence. The data suggest that ST8 CA-MRSA/J has become a successful native clone in Japan, in association with not only SSTIs but also severe invasive infections (posing a threat), requiring attention.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), which possesses staphylococcal cassette chromosome *mec* (SCC*mec*), has been a common nosocomial pathogen since 1961 [1–4]. Another class of MRSA, designated community-acquired MRSA (CA-MRSA), emerged in the community from 1997 to 1999 and has become a major concern worldwide [1, 2, 4, 5]. CA-MRSA generally possesses SCC*mec* type IV (or V), which is suitable for community spread [5, 6], and often produces Panton–Valentine leukocidin (PVL), a toxin acting against polymorphonuclear neutrophils (PMNs), monocytes, and lung tissues [5, 7–9]. CA-MRSA is genetically heterogeneous, with multilocus sequence types (STs) such as ST8, ST30, ST59, and ST80 [5, 7]. CA-MRSA spreads among otherwise healthy children or adolescents, such as athletes, and is primarily associated with skin and soft tissue infections (SSTIs), including pyogenic skin infection, and also with severe systemic infections, such as sepsis, necrotizing pneumonia, necrotizing fasciitis, and osteomyelitis [1, 2, 4].

In the United States, CA-MRSA USA300, with the genotype ST8/*spa*1(t008)/*agr*1/SCC*mec*IVa, is currently the most prominent clone, affecting the entire United States [5]. USA300 is PVL positive and possesses the unique linkage of SCC*mec*IVa and arginine catabolic mobile element (ACME), which confers on its host not only drug resistance but also high virulence/colonization abilities [5, 6, 10]. Peptide cytolysins (such as phenol-soluble modulins, PSMs) have increasingly been noted as virulence factors of CA-MRSA (USA300) [5].

In this study, we investigated the successful Japanese ST8 CA-MRSA clone (ST8 CA-MRSA/J) in terms of community spread and infections, genetic diversity, and comparative genomics (ST8 CA-MRSA/J vs. USA300), aiming at the search for the key factors contributing to ST8 CA-MRSA/J spread and infections.

Cases, materials, and methods

Cases

Infections and colonization by ST8 CA-MRSA/J (including variants) are summarized in Table 1. CA-MRSA and hospital-acquired MRSA (HA-MRSA) were classified according to a previous definition [14]. Severe invasive infections are as follows. In one case (case 1), an 11-year-old boy contracted influenza A on February 1, 2008, and had fever and back pain from February 8. He was hospitalized on February 15 because of fever (39.2°C) and severe back pain extending from the thoracic vertebrae to the lumbar vertebrae. White blood cell (WBC) count and C-reactive protein (CRP) were 20,100/μl and 17.5 mg/dl, respectively. Multiple abscesses at the erector spinea muscles and the epidural region of the spine extending from T8 to L4 were observed. MRSA was detected upon blood culture, biopsy specimen of the lesion, and abscesses; his CA-MRSA was termed NN50. He underwent drainage, and vancomycin (2 g a day) was administered.

In the other case (case 2), a 15-year-old boy (soccer player) became ill with influenza B on March 14, 2007. A feeling of general malaise continued, and he started experiencing right chest pain from the beginning of April. He had fever (38°C), bloody sputum, and progressive dyspnea on April 18, and was admitted to a hospital on April 19. Chest radiography revealed permeation shadows with multiple cavities in the right middle lung. WBC count and CRP were 15,000/μl and 5.8 mg/dl, respectively. MRSA was detected upon culture of bloody sputum; his CA-MRSA was termed 3457. His necrotizing pneumonia was treated with teicoplanin (800 or 400 mg/day), clindamycin (1.8 g/day), and panipenem (1.5 or 1.0 g/day).

Bacterial strains

Table 1 lists 25 strains of ST8 CA-MRSA/J (including variants). Study A includes epidemiologically defined community infections ($n = 13$). Study B1 includes a nosocomial infection, and study B2 includes non-multidrug-resistant ST8 (or single locus variant) MRSA strains ($n = 11$) from hospitals; ST8 (or variant) accounted for 34.4% (11/32) of non-multidrug-resistant strains ($n = 32$) isolated in hospitals [most HA-MRSA strains (>90%) were multidrug resistant]. CA-MRSA strains from a Japanese family in Hong Kong were provided by Pak-Leung Ho [13]. Environmental ST8 MRSA strains ($n = 3$) were isolated from the straps and handrails of trains from 2008 to 2011 in Tokyo and Niigata. ST8 MRSA accounted for 33.3% (5/15) of MRSA train isolates ($n = 15$), which included strains described previously [15]. USA300 included type strain (USA300-0114; this strain was kindly provided by L.K. McDougal and L.L. McDonald) and three Japanese isolates: NN36 from a 3-month-old Indian girl with abscesses [16]; NN47 from an 11-month-old Japanese girl with cellulitis and sepsis followed by osteomyelitis [17]; and 549 from an inpatient and medical staff with abscesses and cellulitis [18]. PVL-positive CA-MRSA included ST22 strain NN48 from familial infection cases [19], ST30 strains NN1 and NN12 from bullous impetigo

Table 1 Clinical and bacteriological information on ST8 community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA)/J (including variants) isolated in the community (study A) and in hospitals (study B)

Strains (ST type)	Age	Sex	MRSA-associated disease	Chemotherapy for MRSA infection	Outcome	Admission or outpatient	Isolation year	Epidemiological classification	Isolation place	Source or reference
Study A										
NN3 (ST8)	1 year	M	Bullous impetigo	Fosfomycin	Recovery	Outpatient	2003	CA-MRSA	Niigata	[11]
NN4 (ST8)	7 months	M	Bullous impetigo	Cefdinir	Recovery	Outpatient	2003	CA-MRSA	Niigata	[11]
S2 (ST8)	11 years	M	Colitis	Levofloxacin	Recovery	Outpatient	2004	CA-MRSA	Oita	This study
NN19 (ST8)	8 years	F	Bullous impetigo	Cefditoren	Recovery	Outpatient	2005	CA-MRSA	Niigata	[12]
2A3 (ST8)	7 years	F	Eczema	ND	Recovery	Outpatient	2005	CA-MRSA	Hong Kong	[13]
2F4 (ST8)	2 years	F	Abscess	ND	Recovery	Outpatient	2005	CA-MRSA	Hong Kong	[13]
2F5 (ST8)	30 years	F	Colonization (nares)	–[a]	–[b]	–[c]	2005	CA-MRSA	Hong Kong	[13]
2I5 (ST8)	31 years	M	Colonization (nares)	–[a]	–[b]	–[c]	2005	CA-MRSA	Hong Kong	[13]
TI-5 (ST8)	21 years	M	Atopic dermatitis	Levofloxacin	Recovery	Outpatient	2006	CA-MRSA	Tokyo	This study
3457 (ST8)	15 years	M	Necrotizing pneumonia	Panipenem, teicoplanin, clindamycin	Recovery	Admission	2007	CA-MRSA	Kumamoto	This study
S3 (ST8)	16 years	F	Colitis	Levofloxacin	Recovery	Outpatient	2007	CA-MRSA	Oita	This study
NN39 (ST8)	1 year	M	Bullous impetigo	Cefdinir		Outpatient	2008	CA-MRSA	Niigata	This study
NN50 (ST8)	11 years	M	Sepsis, paravertebral muscle/epidural abscesses	Vancomycin	Recovery	Admission	2008	CA-MRSA	Hyogo	This study
Study B1										
NN44 (ST1334)	15 years	M	Postsurgical infection	Vancomycin, linezolid	Recovery	Admission	2008	Nosocomial infection	Niigata	This study
Study B2										
809 (ST8)	5 months	M	Colonization (urine)	–[a]	–[b]	Outpatient	2008	–[d]	Tokyo	This study
571 (ST8)	1 month	F	Colonization (sputum)	–[a]	–[b]	Outpatient	2009	–[d]	Tokyo	This study
737 (ST8)	1 month	F	Colonization (sputum)	–[a]	–[b]	Outpatient	2009	–[d]	Tokyo	This study
3826 (ST8)	3 months	F	Colonization (sputum)	–[a]	–[b]	Outpatient	2009	–[d]	Tokyo	This study
5184 (ST new)	51 years	M	Cellulitis	Vancomycin	Recovery	Admission	2009	–[d]	Tokyo	This study
5135 (ST8)	3 years	M	Pneumonia	Fosfomycin	Recovery	Admission	2009	–[d]	Tokyo	This study
5320 (ST new)	86 years	F	Cellulitis	Vancomycin	Recovery	Admission	2009	–[d]	Tokyo	This study
6400 (ST8)	3 years	M	Colonization (sputum)	–[a]	–[b]	Outpatient	2009	–[d]	Tokyo	This study
9946 (ST8)	70 years	M	Colonization (nares)	–[a]	–[b]	Admission	2009	–[d]	Tokyo	This study
658 (ST8)	92 years	F	Colonization (sputum)	–[a]	–[b]	Admission	2009	–[d]	Tokyo	This study
3963 (ST8)	84 years	F	Colonization (hydrothorax)	–[a]	–[b]	Admission	2010	–[d]	Tokyo	This study

[a] No chemotherapy

[b] Not followed up

[c] Carrier

[d] Not defined (because of lack of information)

and bloodstream infection [11], ST59 strains PM1 (from Taiwan [20]) and OS7 from cellulitis [21], and ST80 strains HT20030345 (from the Netherlands) and HT200 30442 (France) [22]; strains HT20030345 and HT200 30442 were kindly provided by Jerome Etienne. PVL-negative CA-MRSA included ST88 strain NN51 [19], ST89 strain NN7 [11], and ST91 strain NN15 [11]; ST88, ST89, and ST91 strains were from bullous impetigo and were exfoliative toxin (ET) positive. The New York/Japan clone [major HA-MRSA in Japan, which carried SaPIm1/n1 with the *tst* gene (encoding toxic shock syndrome toxin-1, TSST-1)] included reference strains Mu50 and N315 (which were kindly provided by Keiichi Hiramatsu), and strains I6 and I8 from 56- and 59-year-old patients who developed toxic shock syndrome (TSS) in the postoperative period of gastric cancer [11].

Molecular characterization of MRSA

Molecular typing of MRSA was performed as described previously [20]. It included ST typing, clonal complex (CC) typing, *spa* (protein A gene) typing [using public *spa* type databases, eGenomics (http://tools.egenomics.com/) or Ridom SpaServer (http://spaserver.ridom.de/)], accessory gene regulator (*agr*) typing, SCC*mec* typing [according to the guidelines by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements [23] and Web page (http://www.staphylococcus.net/)], and coagulase (Coa) typing (using a staphylococcal coagulase antiserum kit; Denka Seiken, Tokyo, Japan). The 44 virulence genes were analyzed by polymerase chain reaction (PCR); they included 3 leukocidin genes (including PVL gene), 5 hemolysin genes, 17 staphylococcal enterotoxin (SE) genes (including *tst*), 1 putative SE gene, 3 exfoliative toxin (ET) genes, 14 adhesin genes, and ACME-*arcA* gene [6].

The novel SCC*mec*IVnew.1.1.1 (SCC*mec*IVl) of ST8 CA-MRSA/J [24] was detected by PCR using two primer sets: Fw (5′-TGACCTCCAAGTAACAAAAG) and Rv (5′-TCATCGTTACGTTACTTGGT), generating a 485-bp PCR product [24]; and 25F (5′-AGCCCTTCAACTGTAACCT) and 16R (5′-GTAGTTGCACCAATCGTAGA), generating a 650-bp PCR product. TSST-1 production levels (the amount of TSST-1 in the supernatant of bacterial cultures at $2.0 \times l0^9$ cfu/ml) were examined using a TST-RPLA kit (Denka Seiken). Pulsed-field gel electrophoresis (PFGE) analysis was performed with *Sma*I digestion, as described previously [11].

Assay of the expression levels of virulence genes

The mRNA expression levels of nine virulence genes (including the *psmα* gene) were examined, compared with USA300, as described previously [19]. Primers used were those described previously [20, 25].

Susceptibility testing

Susceptibility testing of bacterial strains was carried out using the agar dilution method with Mueller–Hinton agar according to previous procedures by the Clinical and Laboratory Standards Institute [26].

Pyrosequencing of the MRSA genome and homology analysis

The MRSA NN50 genome was analyzed by pyrosequencing using a genome sequencer FLX system (Roche Diagnostics, Branford, CT, USA). In this study, 190,063 reads yielded 74-Mb raw sequences ($\sim$26 fold of the genome); GenBank accession number for the NN50 genome is BAEA01000000. The mapping of contigs on the 2,822,306-bp USA300 (FPR3757) genome (GenBank accession number NC_007793) and the search for genes or open reading frames (*orf*) were performed using the software in silico MolecularCloning (version 4.2) (In Silico Biology, Yokohama, Japan). GenBank accession numbers for the DNA sequences carrying new SaPI with the *tst*, *sec*, and *sel* genes and gentamicin resistance transposon Tn*4001*, determined in this study, are AB679717 and AB682805, respectively; that for SCC*mec*IVl is AB678405.

Statistical analysis

Data for mRNA expression assay were evaluated by analysis of variance with repeated measurement. The level of significance was defined as a P value <0.05.

Results

Community spread and infections in ST8 CA-MRSA/J

ST8 MRSA clinical strains ($n = 25$) were collected during 2003 and 2010 in Japan and Hong Kong (Table 1). In Japan ($n = 21$), isolates from SSTIs (bullous impetigo, abscesses, cellulitis, atopic dermatitis, and eczema) accounted for 33.3% (7/21); pneumonia for 9.5% (2/21); colitis for 9.5% (2/21); paravertebral muscle/epidural abscesses with sepsis for 4.8% (1/21), postsurgical (wound) infection (4.8%, 1/21); and colonization for 38.1% (8/21). Geographic location covered five prefectures.

Definite ST8 CA-MRSA infections (study A, $n = 13$) included seven SSTI cases, two colitis cases, and two serious invasive cases (necrotizing pneumonia and sepsis/epidural abscesses).

ST8 MRSA was also isolated in hospitals, including postsurgical infection (study B1). It accounted for 34.4% (11/32) in a non-multidrug-resistant MRSA pool (likely CA-MRSA) in hospitals (study B2); the cases were largely colonization ($n = 8$), but included pneumonia ($n = 1$) and cellulitis ($n = 2$).

Moreover, ST8 MRSA spread even to surfaces and subway trains, and was transferred to Hong Kong through a Japanese family (whose members were all colonized or infected) (study A).

Genotypes and diversity in ST8 CA-MRSA/J

For 21 clinical strains in Japan, a major genotype was ST8/*spa*606(t1767)/*agr*1/SCC*mec*IVl/CoaIII, with divergence in ST type, *spa* type (Table 2). All the strains were negative for PVL and ACME and positive for superantigenic toxin-encoding *S. aureus* pathogenicity island SaPI (carrying the *tst*, *sec*, and *sel* genes). TSST-1 production levels of ST8 CA-MRSA/J (strain NN50) were higher than the New York/Japan clone carrying SaPIm1/n1 (even more than strains from TSS patients) (Fig. 1). Most strains were resistant to gentamicin, because of *aacA-aphD* (95.2%, 20/21), and many strains were positive for *sep* (66.7%, 14/21) (Table 2).

The environmental strains (from trains) and Hong Kong strains also showed very similar characteristics to the clinical strains (Table 2).

The above ST8 CA-MRSA/J (and its variants) differed from USA300 in terms of *spa* type, SCC*mec* type, PVL, ACME, SaPI carrying *tst*, *sec*, and *sel*, SaPI carrying *sek* and *seq*, phage carrying *sep*, and gentamicin resistance, as shown in Table 2.

Next, PFGE patterns of ST8 CA-MRSA/J strains were analyzed (Fig. 2). A major PFGE pattern (named ST8Jα) consisted of 11 strains (from SSTIs, colitis, and environments in Tokyo, Niigata, and Oita) and may include an additional 2 strains (from a hospital MRSA pool in Tokyo). PFGE-type ST8Jβ included 2 strains (NN50 and 3457) from severe invasive infections, representing a severe type.

Two strains (2F4 and 2F5) from Hong Kong shared the same PFGE pattern (ST8Jγ) as strains NN3 and S2 in Japan, indicating transmission from Japan to Hong Kong; two additional strains (2I5 and 2A3) from Hong Kong also resembled ST8Jγ. All ST8 CA-MRSA/J strains were divergent from USA300; four USA300 strains slightly diverged from each other by one or two bands (Fig. 2).

Comparative genomics of ST-8 CA-MRSA/J (strain NN50)

The NN50 genome was 97.4% homologous to the USA300 (strain FPR3757) genome, albeit with marked divergence in movable genetic structures (Fig. 3). NN50 lacked the ACME region, SaPI5 (carrying *sek* and *seq*), and phage φSA2usa (carrying the PVL gene) of USA300. Instead, NN50 possessed a novel SaPI carrying *tst*, *sec*, *sel*, and *bla* (named SaPIj50), which was a mosaic structure composed of SaPIm4, PI T0131, and SaPIm1/n1 (Fig. 4). NN50 also carried gentamicin resistance transposon Tn*4001*, and ampicillin resistance transposon Tn*552*. A phage φSa1j was also inserted; φSa1j showed approximately 80% homology to corresponding phages φSa1 (such as φNM1 and φ11). φSa3 in NN50 (named φSa3j) acquired *sep*, but lacked *chp* (encoding chemotaxis inhibitory protein), as shown in Fig. 5a. NN50 had no plasmids.

NN50 possessed SCC*mec*IV with a novel subtype (SCC*mec*IVl) (Fig. 3). The size of SCC*mec*IVl was 25,555 bp, and the 8,193-bp J1 joining region contained a 4.8-kb *orf*, encoding a novel 1,604-amino-acid cell wall-anchored surface protein with the LPXTG motif (named CWASP/J). The CWASP/J gene (*spj*) showed no homology to previous sequences. PCR assay with primer sets (Fw and Rv) and (25F and 16R) (Fig. 3) revealed that all ST8 CA-MRSA/J strains (including variants) in Table 2 carried the *spj* gene (SCC*mec*IVl).

Similarly to USA300, NN50 possessed additional virulence genes and regions such as genomic islands (vSaα to γ) and a series of immuno evasion-related genes, such as major histocompatibility complex class II analog gene (*map*), albeit with divergence (Figs. 3 and 5b).

The mRNA expression levels of the toxin and adhesin genes

The mRNA expression levels of ST8 CA-MRSA/J (strains NN50 and 3457) were examined, and compared with CA-MRSA with other ST types or HA-MRSA (the New York/Japan clone) (Fig. 6). For cytolysin genes (*psm*α, *hld*, and *lukED*), CA-MRSA (including ST8 CA-MRSA/J and USA300) shared very similar expression levels, irrespective of ST types, but their expression levels (especially those for *lukED*) were significantly higher than HA-MRSA. The *hla* and *hlg* expression levels of CA-MRSA tended to be higher than HA-MRSA, but the data were not significantly different (data not shown).

For adhesin genes, *fnb* and *clf* expression levels were similar among CA-MRSA clones but significantly higher than HA-MRSA (Fig. 6).

Discussion

MRSA is a powerful pathogen, which can acquire or exchange virulence and drug resistance genes through horizontal gene transfer or recombination to adapt to

Table 2 Molecular characterization of ST8 CA-MRSA/J strains (including variants) isolated in Japan and Hong Kong, compared with USA300 strains isolated in the United States and Japan

Type, virulence gene, drug resistance	Japan		Hong Kong	USA300		
	Clinical strains[a] ($n = 21$)	Environmental strains ($n = 3$)	Family strains ($n = 4$)	USA300 -0114	USA300 -FPR3757	Isolates in Japan[b] ($n = 3$)
Type						
CC	8	8	8	8	8	8
ST	8 (85.7%), 1334 (4.8%), new (9.5%)	8	8	8	8	8
spa	606 (t1767) (76.1%), (t008) (9.5%), 364 (t622) (4.8%), 605 (new) (4.8%), new (t9102) (4.8%)	606 (t1767) (1/3), new (t986) (2/3)	606 (t1767) (1/4), new (t1766) (1/4), new (t1747) (2/4)	1 (t008)	1 (t008)	985 (t711) (1/3), 363 (t024) (1/3), 1 (t008) (1/3)
agr	1	1	1	1	1	1
SCC*mec* type	IV.new.1.1 (IVl)[c]	IV.new.1.1 (IVl)[c]	IV.new.1.1 (IVl)[c]	IVa	IVa	IVa
Coagulase type	III	III	III	III	ND	III
Virulence gene						
Leukocidin						
luk$_{PV}$SF	−	−	−	+	+	+
lukE-lukD	+ (76.2%)	+	+	+	+	+
lukM	−	−	−	−	−	−
Hemolysin						
hla, hlg, hlg-v, hld	+	+	+	+	+	+
hlb	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]
Enterotoxin						
SaPI (*tst, sec, sel*)	+	+	+	−	−	−
sep	+(66.7%)	+	+(1/4)	−	−	−
egc (*seg, sei, sem, sen, seo*)	−	−	−	−	−	−
sea, seb, sed, see, seh, sej, set, seu	−	−	−	−	−	−
SaPI5 (*sek, seq*)	−	−	−	+	+	+
Exfoliative toxin						
eta, etb, etd	−	−	−	−	−	−
Other						
edin	+ (52.4%)	+	+ (2/4)	−	−	−
Adhesin						
c12ag[e]	+	+	+[f]	+	+	+
cna, bbp	−	−	−	−	−	−
ACME-*arcA*	−	−	−	+	+	+
Drug resistance	G (95.2%), K (95.2%), E (14.3%)	G, K	G (3/4), K, T (1/4)	K, E, T, N	E, T, M	K (2/3), E, N (1/3)

Table 2 continued

Type, virulence gene, drug resistance	Japan		Hong Kong	USA300		
	Clinical strains[a] ($n = 21$)	Environmental strains ($n = 3$)	Family strains ($n = 4$)	USA300 -0114	USA300 -FPR3757	Isolates in Japan[b] ($n = 3$)
Plasmid	55 kb (2/5), 50 kb (1/5)–(2/5)	55 kb (2/2)	ND[g]	32 kb, 3.0 kb	37 kb, 4.4 kb, 3.1 kb	35 kb (1/3), 32 kb (1/3), 3.0 kb (1/3), 1.4 kb (1/3)

CA-MRSA community-acquired methicillin-resistant *Staphylococcus aureus*, *G* gentamicin, *K* kanamycin, *T* tetracycline, *E* erythromycin, *N* norfloxacin, *M* mupirocin

[a] Including strain NN50, which exhibited ST8 and *spa*606 (t1767); was positive for *lukE-lukD*, *sep*, and *edin;* was resistant to gentamicin; and lacked plasmids

[b] NN36 from Tokyo in 2007 (16), NN47 from Saitama in 2008 (17), 549 from Okinawa in 2008 (18)

[c] SCC*mec*IV.new.1.1 was tentatively designated SCC*mec*IVl (15)

[d] Split *hlb* gene caused by insertion of bacteriophage

[e] *c12ag*, core 12 adhesin genes shared by all strains: *icaA*, *icaD* (for biofilm formation); *eno* (for laminin-adhesin); *fnbA*, *fnbB* (for fibronectin-adhesin); *ebpS* (for elastin-adhesin); *clfA*, *clfB*, *fib*, *sdrC*, *sdrD*, *sdrE* (for fibrinogen-adhesin)

[f] Includes one *fib*-negative case

[g] Not determined

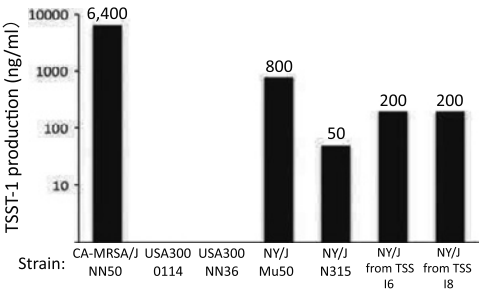

Fig. 1 TSST-1 production levels of ST8 community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA)/J (strain NN50), compared with USA300 and the New York/Japan clone. USA300 lacks the *tst* gene encoding TSST-1 (USA300 strains produced no TSST-1). Strains I6 and I8 were isolated from toxic shock syndrome (TSS) patients in Niigata. *NY/J*, New York Japan clone. Typical results in triplicate experiments are shown

hospital or community environments. The year 2011 marks the 50th anniversary of the first report on MRSA, but MRSA has still posed serious threat and claimed many lives. Epidemic MRSA includes divergent clones, and ST8 CA-MRSA USA300 (the most prevalent clone in the United States [5]) is one of the best characterized MRSA. However, the contribution of PVL and ACME in the USA300 virulence, for example, remains to be conclusively proven, and the key virulence factors contributing to USA300 community spread and virulence are still not fully understood [5]; for instance, the role of PVL in the pathogenesis of MRSA infections is controversial in animal models, although PVL may exert strong activity on human neutrophils [9]. In this study, we characterized ST8 CA-MRSA/J (a successful native clone in Japan), clinically and genetically (at the genome levels) for the first time, compared with ST8 CA-MRSA USA300.

Regarding SSTIs caused by ST8 CA-MRSA/J, deep pyogenic skin infections with severe pain and extremely large abscesses were rare, in contrast to PVL-positive CA-MRSA (including USA300) [1, 4, 16, 18, 19], which may be the result of lack of PVL in ST8 CA-MRSA/J. Bullous impetigo is a common disease among children [4, 27], and ET-producing *S. aureus* is a major cause [27]. Although ST8 CA-MRSA/J was ET negative, it was frequently isolated from bullous impetigo. Bullous impetigo could be caused by a mixed infection with ET-positive *S. aureus* [12]. In Japan, MRSA is isolated from 10–20% of *S. aureus* from bullous impetigo [11, 12, 28]; the ST8 type accounted for approximately 16.2% (11/68) of such MRSA cases (unpublished data). It is strongly speculated that ST8 CA-MRSA/J infects scratched skin and soft tissues in children using a possible adhesin, CWASP/J (encoded by the *spj* gene of SCC*mec*IVl).

Athletes are at risk for CA-MRSA infections through skin-to-skin contact [4, 29]. In this study, two cases were student athlete infections by ST8 CA-MRSA/J.

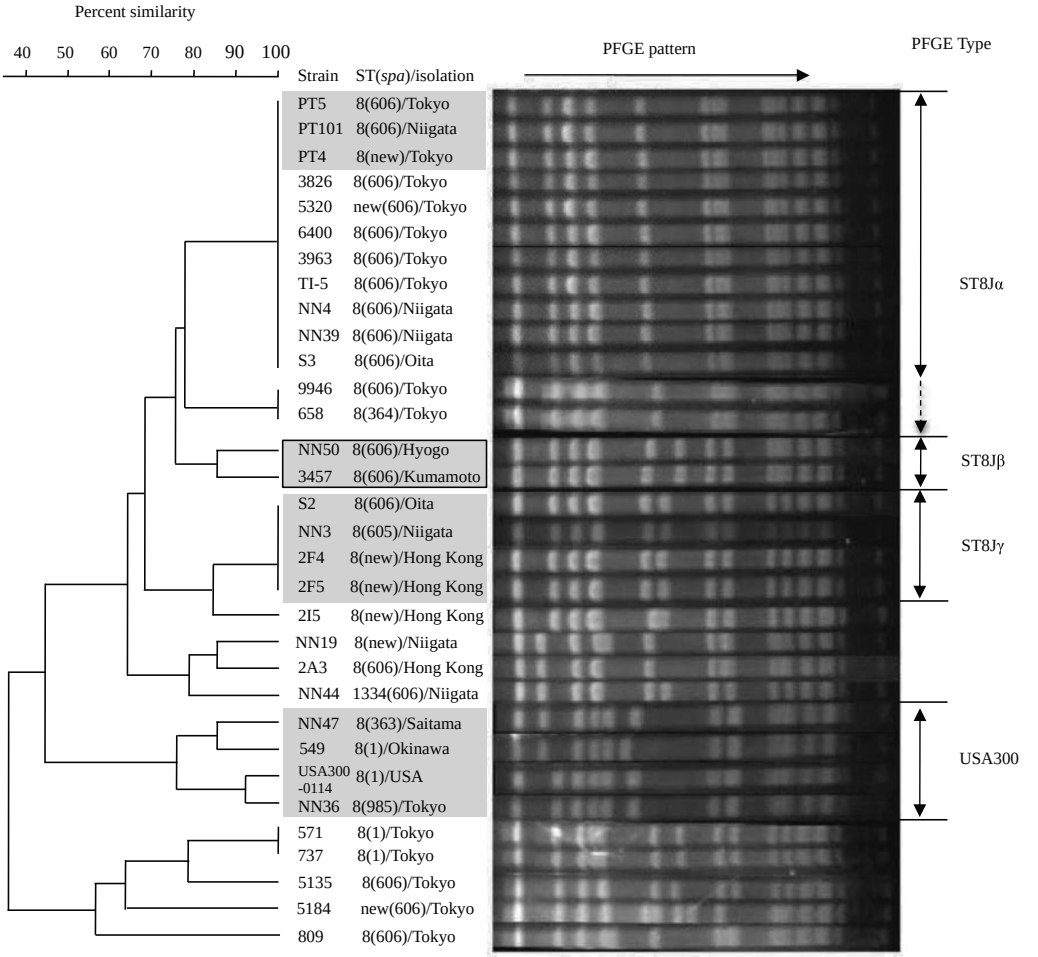

Fig. 2 Pulsed-field gel electrophoresis (PFGE) patterns of ST8 CA-MRSA/J strains (including variants). Public transport strains, severe invasive infection cases, Hong Kong strains directly related to strains in Japan, and USA300 strains are all *shaded* separately; a severe invasive infection case is marked in *black*

For enteritis, because severe MRSA enteritis was noted in the 1980s to 1990s in Japan, when hospital MRSA outbreaks occurred [30], and superantigens, such as TSST-1, were noted as important virulence factors, ST8 CA-MRSA/J enteritis could be caused most probably by higher levels of TSST-1 (or combination of superantigens).

Invasive infections were also characteristics of ST8 CA-MRSA/J and included uncommon epidural abscesses with sepsis and necrotizing pneumonia with severe and rapid cavity formation. Responsible virulence factors may include α-hemolysin (Hla) and cytolytic peptides (Psms and Hld), which were proposed by Diep and Otto [5]. TSST-1, which was produced at much higher levels than the New York/Japan clone, may also contribute to the ST8 CA-MRSA/J pathogenesis. Interestingly, the PFGE type of these ST8 CA-MRSA/J invasive strains was the same (ST8J*β*), representing a virulent type.

For USA300, community-acquired pneumonia is noted during the influenza season [31]. In this study, patients with severe invasive infections had suffered from influenza 1 week before the MRSA-related symptoms, suggesting an influenza prodrome case, requiring attention to ST8 CA-MRSA/J invasive infection during the influenza season.

From a genetic point of view, ST8 CA-MRSA/J is a geographic variant of ST8 CA-MRSA, which is one of the most worldwide-disseminated lineages [5]. ST8 CA-MRSA/J was isolated in 2003 in Japan [11] for the first time, to our knowledge, and has already undergone clonal expansion,

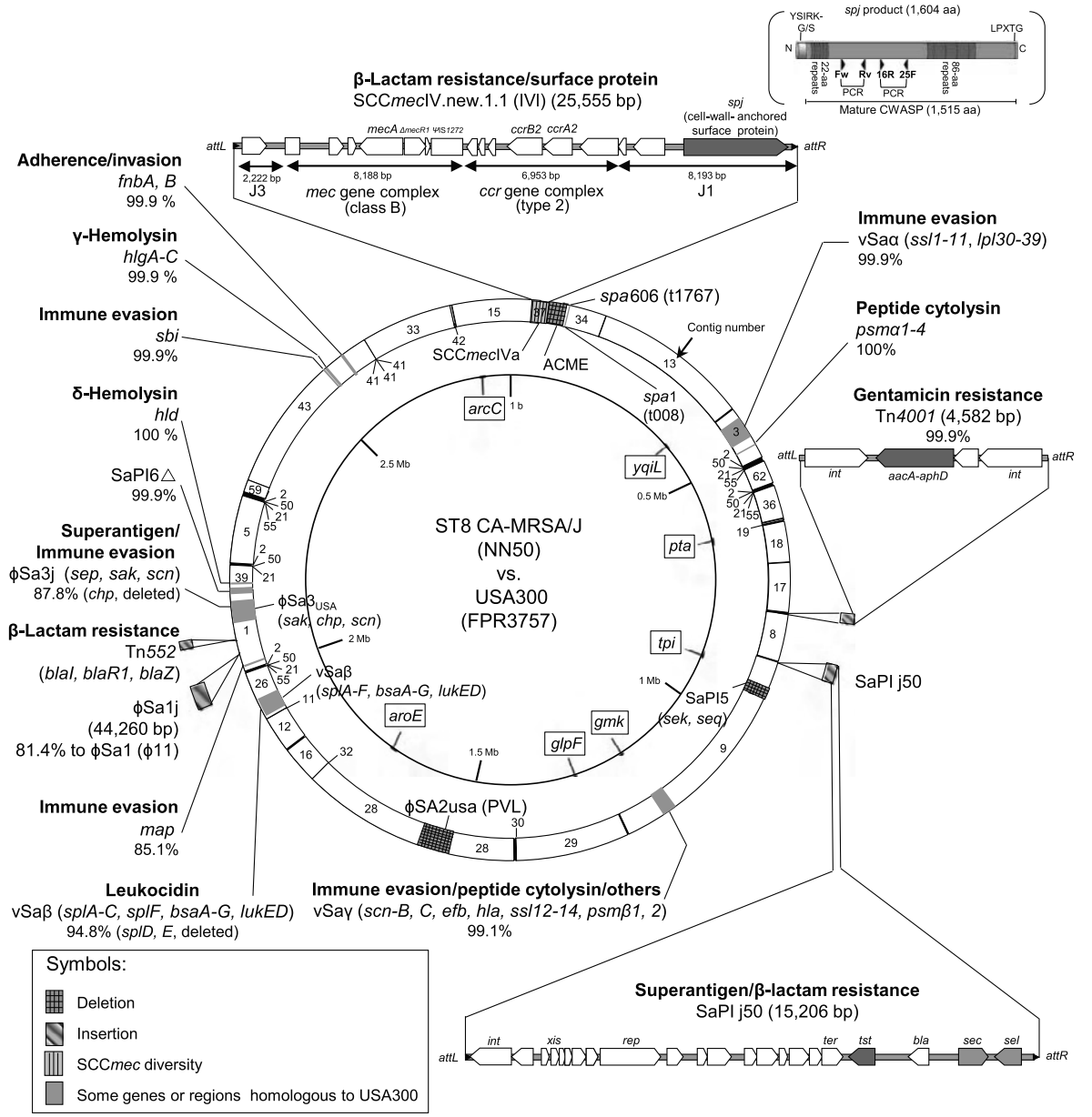

Fig. 3 The genome of ST8 CA-MRSA/J (strain NN50) that was constructed based on the USA300 (FPR3757) genome information. Information on NN50 and USA300 genomes is presented outside and inside the genome *circle*, respectively; for instance, NN50 carried new SCC*mec*IV (SCC*mec*IVl), instead of SCC*mec*IVa in USA300. The structure of CWASP/J encoded by the *spj* gene in SCC*mec*IVl and the location of PCR primer sets (Fw and Rv) and (16R and 25F), to specifically detect the *spj* gene, are also indicated in the figure (*upper right side*). Moreover, NN50 lacked ACME and *φ*SA2usa (carrying the PVL gene), which were present in USA300. NN50 carried genes and regions homologous to USA300, including immunoglobulin (Ig/Fc)-binding protein (protein A) gene (*spa*); a series of peptide cytolysin genes (phenol-soluble modulin genes, *psmα* and *psmβ*); leukocidin and hemolysin genes (such as *lukED* and *hla*); a series of immuno evasion-related genes, such as superantigen-like protein genes (*ssl*), extracellular complement-binding protein/ inhibitor genes (*scn*), major histocompatibility complex class II analog gene (*map*), staphylokinase gene (*sak*), and second Ig-binding protein gene (*sbi*); serine protease genes (*spl*); and bacteriocin genes (*bsa*). *Percentage* represents homology to the corresponding gene or region in USA300

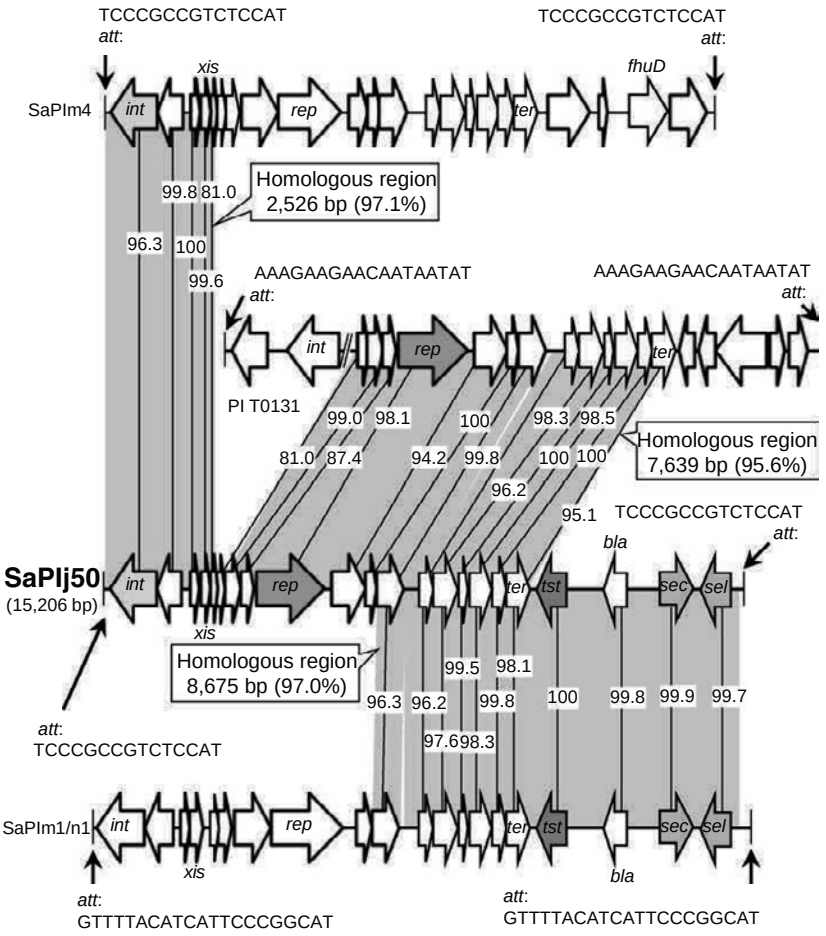

Fig. 4 The mosaic structure of SaPIj50 carrying the superantigen genes, *tst*, *sec*, and *sel*, compared with SaPIm4, PI T0131, and SaPIm1/n1. The GenBank accession numbers of (SaPIm4 and SaPIm1/n1) and PI T0131 are NC_002758 and CP002643, respectively. Homologous regions are *shaded* in the figure (the sizes and percentage homologies of each region are also indicated). The *att* sequence of SaPIj50 was the same as SaPIm4, but divergent from SaPIm1/n1; the left-side region carrying the integrase gene (*int*) of SaPIj50 showed a high similarity to SaPIm4, but not to SaPIm1/n1. The replication initiator gene (*rep*) region of SaPIj50 showed a high similarity to PI T0131, but not to SaPIm4 or SaPIm1/n1. The terminase (which cleaves multimeric DNA) gene (*ter*) showed a similarity to both SaPIm1/n1 and PI T0131. Also, the right-side virulence region carrying the superantigen genes (*tst*, *sec*, and *sel*) showed a high similarity to SaPIm1/n1, indicating that the superantigen region of SaPIj50 originates in SaPIm1/n1 of the New York/Japan clone (the most prominent HA-MRSA in Japan)

growing as a major MRSA in the community in Japan. ST8 CA-MRSA/J includes ST and *spa* variants, and exhibits divergence in terms of carriage of the *lukE-lukD*, *sep*, *edin* genes, drug resistance patterns, and PFGE patterns.

The most exceptional features of the ST8 CA-MRSA/J genome are carriage of novel SCC*mec*IVl and mosaic SaPIj50. SCC*mec*IVl carried the J1 region with the *spj* gene encoding new CWASP with an LPXTG motif (CWASP/J). SCC*mec*-associated CWASP gene (*pls*, for plasmin-sensitive surface protein) was previously reported [32], but the *spj* gene showed no homology to the *pls* gene. CWASPs include adhesins such as collagen adhesin and fibronectin-binding protein A [33], suggesting a possible role of SCC*mec*IVl as a colonization stimulation (because of the *spj* gene in the J1 region of SCC*mec*IVl), in addition to drug resistance suitable for the community (because of SCC*mec*IV) [6]. The origin of the *spj* gene remains unknown.

SaPI is a phage-related chromosomal island, which represents phage satellites producing phage-like infectious particles [34]. SaPIj50 was a novel mosaic structure that most probably emerged through recombination of SaPIm4, PI T0131, and SaPIm1/n1; i.e., the superantigen (*tst*, *sec*, and *sel*) region of SaPIj50 most probably originated from SaPIm1/n1 of the New York/Japan clone (the prominent HA-MRSA clone) in Japan. This finding also indicates that the evolution of ST8 CA-MRSA/J includes the acquisition of the virulence genes of HA-MRSA in Japan.

Based on these evolutionary features, we strongly consider that SCC*mec*IVl and SaPIj50, both of which emerged in and were unique to Japan, must serve as a key factor for ST8 CA-MRSA/J spread and infections in community settings in Japan.

As for superantigens, this notion may be emphasized by the fact that the number of superantigen genes is greater in ST8 CA-MRSA/J than in USA300 (4 vs. 2; ST8 CA-MRSA/J carried SaPIj50 carrying *tst*, *sec*, and *sel* and φSa3 carrying *sep* [35], whereas USA300 carried only SaPI5 carrying *sek* and *seq*) and ST8 CA-MRSA/J is a high TSST-1 producer. TSST-1 may suppress the

mobility of PMNs to infection sites [36], allowing MRSA to invade tissues, in addition to exhibiting toxic shock activities [37]. It should be noted that USA300 and the New York/Japan clone in the United States [38] lack the *tst* gene.

Moreover, ST8 CA-MRSA/J carried common virulence genes on the chromosome, such as the peptide cytolysin genes (*psmα* or *psmβ*) [5, 39], fibronectin binding protein (*fnb*) gene [40], and a series of genes responsible for host immune evasion, such as *map*, *ssl*, and *scn* [41, 42], albeit with divergence (e.g., repetitions in *map*).

Finally, ST8 CA-MRSA/J became gentamicin resistant by the acquisition of transposon Tn*4001* [43] on the chromosome. In Japan, CA-MRSA generally exhibits gentamicin resistance at high frequency (80–90%, [11]; unpublished data), because gentamicin ointment has commonly been used for the treatment of SSTIs. Gentamicin resistance is occasionally encoded by a plasmid [11].

In conclusion, although ST8 CA-MRSA/J is a geographic variant of the ST8 CA-MRSA lineage, it now has become well adapted to the Japanese community as a successful native CA-MRSA clone, as characterized by the carriage of SCC*mec*IVl, SaPIj50, and Tn*4001*. Its evolution included a salvage step (through recombination) of the virulence region of the HA-MRSA New York/Japan clone. ST8 CA-MRSA/J has undergone clonal expansion. We speculate that the unique combination (in synergy) of virulence factors (such as CWASP/J, TSST-1, PSMs, α-hemolysin, and others) and drug (e.g., gentamicin) resistance allows ST8 CA-MRSA/J to be a successful clone.

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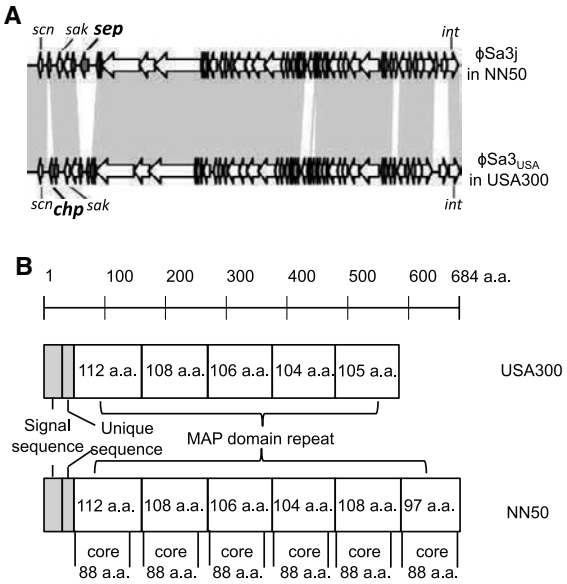

Fig. 5 Divergence between the structures of φSa3 (**a**) or major histocompatibility complex (MCH) class II analog (MAP) (**b**) in ST8 CA-MRSA/J strain NN50 and USA300. GenBank accession number of φSa3usa and Map in USA300 is NC_007793. In **a**, homologous regions are *shaded*. In **b**, for *map*, the number of repeated domains of 110 residues (which include a 31-amino-acid subdomain with a high similarity to the peptide-binding groove of the β-chain of MHC class II proteins) was five times for USA300 and six times for NN50 (indicating more repetitions in NN50)

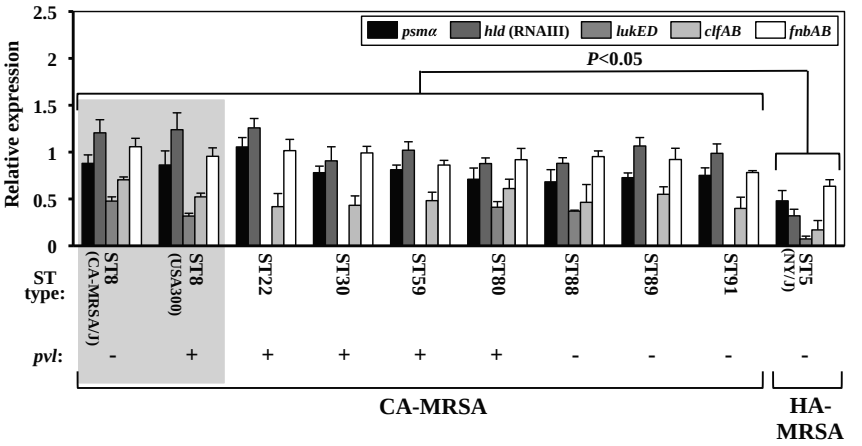

Fig. 6 The levels of mRNA expression for the virulence genes in ST8 CA-MRSA/J, compared with other CA-MRSA and hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA). Bacterial strains are described in "Cases, materials, and methods." The data of ST8 CA-MRSA/J were made of two strains, NN50 and 3457. The data of PVL-positive and PVL-negative CA-MRSA groups (including USA300 and ST8 CA-MRSA/J) were similar, but they were significantly higher than the New York/Japan (NY/J) clone of HA-MRSA ($P < 0.05$). The PVL gene expression levels were similar among the PVL-positive clones (data not shown). The ST22, ST30, ST59, ST89, and ST91 clones were negative for the *lukED* genes

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