Identification of different clonal complexes and diverse amino acid substitutions in penicillin-binding protein 2 (PBP2) associated with borderline oxacillin resistance in Canadian *Staphylococcus aureus* isolates

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Borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) exhibit oxacillin MIC values of 1–8 µg ml⁻¹, but lack *mecA*, which encodes the low-affinity penicillin-binding protein (PBP)2a. The relationship of the BORSA phenotype with specific genetic backgrounds was assessed, as well as amino acid sequence variation in the normal PBP2. Among 38 BORSA, 26 had a common PFGE profile of genomic DNA, and were multilocus sequence type (ST)25. The other isolates were genetically diverse. Complete *pbp2* sequences were determined for three BORSA, corresponding to ST25, ST1 and ST47, which were selected on the basis of lacking *blaZ*-encoded β-lactamase. The essential transpeptidase-domain-encoding segment of *pbp2* was also sequenced from seven additional ST25 isolates. Amino acid substitutions occurred in the transpeptidase domain of all BORSA, irrespective of clonal type. A Gln₆₂₉→Pro substitution was common to all ST25 BORSA, but most could be distinguished from one another by additional unique substitutions in the transpeptidase domain. The ST1 and ST47 isolates also possessed unique substitutions in the transpeptidase domain. Plasmid-mediated expression of *pbp2* from an ST25 or ST1 isolate in *S. aureus* RN6390 increased its oxacillin MIC from 0.25 to 4 µg ml⁻¹, while *pbp2* from a susceptible strain, ATCC 25923, had no effect. Therefore, different amino acid substitutions in PBP2 of diverse BORSA lineages contribute to borderline resistance. The predominant ST25 lineage was not related to any of the five clonal complexes that contain methicillin-resistant *S. aureus* (MRSA), suggesting that ST25 cannot readily acquire *mecA*-mediated resistance.

Received 26 April 2006
Accepted 8 August 2006

INTRODUCTION

Staphylococcus aureus is a leading cause of nosocomial infections, and is distinguished by its ability to cause infection in virtually every tissue and organ system of the body (Archer, 1998). Therefore, rapid eradication of *S. aureus* is of paramount importance in the treatment and

control of nosocomial infections. However, the introduction of penicillin into clinical practice was quickly followed by the appearance of β-lactamase-mediated resistance. The introduction of β-lactamase-resistant semi-synthetic forms of penicillin was also quickly followed by the acquisition of an alternative penicillin-binding protein (PBP), PBP2a, encoded by *mecA* on the staphylococcal cassette chromosome SCC_{mec} (Hiramatsu *et al.*, 2001; Ito *et al.*, 2001). Methicillin-resistant *S. aureus* (MRSA) are resistant to all members of the β-lactam family, due to the low affinity of PBP2a for β-lactams (Hartman & Tomasz, 1984). As endemic hospital-associated MRSA are typically resistant to multiple antimicrobial agents, an increased emphasis has been placed on their rapid identification, both for determining an appropriate antimicrobial regimen, and to enable appropriate infection control measures (Louie *et al.*, 2000; van Griethuysen *et al.*, 2001; Yamazumi *et al.*, 2001). A

Abbreviations: BORSA, borderline oxacillin-resistant *S. aureus*; CC, clonal complex; MLST, multilocus sequence typing; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; PBP, penicillin-binding protein; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers of *pbp2* sequences determined from strains MA15 and MSH12 in this study are AF540019 and AF540020, respectively. Accession numbers AF540021–AF540028 were assigned for the respective partial nucleotide sequences of *pbp2* from additional BORSA isolates MA4, MA6, MA8, MA9, MA13, MA14, MSH7 and OGH2.

by-product of the quest for more sensitive diagnostic techniques has been the identification of strains with an intermediate level of resistance, known as borderline oxacillin-resistant *S. aureus* (BORSA) (Varaldo, 1993; Varaldo *et al.*, 1993).

Although BORSA infections can be treated with β -lactams (Varaldo, 1993), outbreaks continue to occur (Balslev *et al.*, 2005); BORSA have been associated with both community-acquired and nosocomial infections in multiple institutions, and have been isolated from various infection sites, including skin and surgical wounds (Kernodle *et al.*, 1998; Massanari *et al.*, 1988; Suggs *et al.*, 1999). Although BORSA do not exhibit a single trait that differentiates them from fully susceptible or resistant strains, many share a common 94/96 phage type, a high degree of genetic relatedness, and a 17.2 kb plasmid that confers hyper-production of β -lactamase (Barg *et al.*, 1991; Massidda *et al.*, 1996; McMurray *et al.*, 1990). This last feature has been proposed as a factor that contributes to the BORSA phenotype (Barg *et al.*, 1991; McDougal & Thornsberry, 1986), as well as a novel meticillinase (Keseru *et al.*, 2005; Massidda *et al.*, 1992). Others have noted that spontaneous amino acid substitutions in PBP2 of *S. aureus* can be induced by selection for growth in the presence of ceftizoxime (Leski & Tomasz, 2005), and different substitutions in PBP2 have been noted in one study conducted with a single clinical BORSA strain, CDC-6 (Hackbarth *et al.*, 1995).

Although these archetypal BORSA share a high degree of genetic relatedness, several issues are unresolved, including their association with the overall population structure of *S. aureus*, which is predominantly clonal (Feil *et al.*, 2003, 2004; Feil & Enright, 2004), the extent to which amino acid substitutions in PBP2 from the BORSA strain CDC-6 are shared by other related BORSA, and the presence of amino acid substitutions in PBP2 from BORSA strains that do not conform to the prototypic 94/96 phage type strains represented by CDC-6. In this respect, diverse amino acid substitutions within the equivalent of PBP2 in *Streptococcus pneumoniae* also promote reduced susceptibility to β -lactams (Asahi *et al.*, 1999; Mouz *et al.*, 1999). However, the genetic competence of *Strep. pneumoniae* and its capacity for interspecies recombination have contributed to a mosaic structure of PBP in penicillin-resistant strains (Dessen *et al.*, 2001; Laible *et al.*, 1991), whereas *S. aureus* is not noted for genetic competence (Tortosa & Dubnau, 1999), and is more likely to alter gene function by point mutation than genetic recombination (Feil *et al.*, 2003, 2004; Feil & Enright, 2004). Herein, we report on the clonal associations of genetically diverse BORSA, an analysis of amino acid substitutions in their PBP2s, and the contribution of the latter to the BORSA phenotype.

METHODS

Bacterial strains, plasmids and growth media. Regional health-care centres from across Canada were requested to submit *S. aureus* strains that exhibited an oxacillin MIC ≥ 1 and ≤ 8 $\mu\text{g ml}^{-1}$,

but were *mecA* negative. All strains that satisfied these criteria were confirmed as *S. aureus* which lacked *mecA*, using a multiplex PCR assay that detected both the nuclease gene (specific for *S. aureus*) and *mecA*, with detection of bacterial 16S rRNA as an internal control, as described previously (Louie *et al.*, 2002). Control organisms used with each batch of samples tested included *S. aureus* ATCC 43300 (MRSA) and meticillin-susceptible *S. aureus* (MSSA) ATCC 29213. By these criteria, 38 BORSA were collected over a period of 8 years (1990–1998). The isolates were from different geographic locations in Quebec, Ontario and Manitoba, and deemed to be epidemiologically unrelated, as evident either from the identification of distinct genetic backgrounds or from the existence of strains of a similar genetic background that originated from different locations or non-overlapping time spans.

Confirmed BORSA were subjected to antibiotic-susceptibility testing, using cation-adjusted Mueller–Hinton broth supplemented with 2% NaCl, unless otherwise indicated, according to standard guidelines (National Committee for Clinical Laboratory Standards, 2003). Phage typing and PFGE of *Sma*I-digested genomic DNA were conducted as described previously (Simor *et al.*, 2001, 2002). Additional strains and plasmids used in this study are described in Table 1. *S. aureus* was grown at 37 °C in brain heart infusion (BHI) broth (Difco). *Escherichia coli* was grown at 37 °C in Luria–Bertani medium (Difco). Media were supplemented with chloramphenicol (10 $\mu\text{g ml}^{-1}$) or ampicillin (50 $\mu\text{g ml}^{-1}$), where appropriate.

Plasmid isolation and Southern hybridization. Plasmid isolation from either *E. coli* or *S. aureus* was performed using reagents provided with the GenElute plasmid kit (Sigma), using a lysostaphin treatment step for *S. aureus*, as necessary. Southern hybridization of *Hind*III-digested genomic DNA was performed as described previously (Rice *et al.*, 2001), using the Amersham ECL Direct chemiluminescence labelling system. A 316 bp probe representing the 3' end of the *S. aureus blaZ*-coding sequence was generated by PCR with the forward and reverse primers 5'-TTCAACACCTGCTGCTTTC-3' and 5'-CACTCTTGGCGGTTTAC-3', using genomic DNA of *S. aureus* ATCC 29213 as template.

Cloning and sequencing of *pbp2*. The *pbp2* gene is preceded by *prfA* (Pinho *et al.*, 1998), encoding a putative DNA recombination and repair function, and the two genes can be transcribed independently or as a polycistronic RNA originating from the *prfA* promoter. To ensure optimal expression, PCR of genomic DNA was employed to amplify a 3.2 kb fragment, containing *pfrApbp2*, using forward and reverse primers 5'-cgggacccCACATACTTGTACTTGCCTC-3' and 5'-cccagagctcTTTATGTTGAGTGGGA-3', respectively. A 1.28 kb fragment spanning the transpeptidase domain (amino acids 278–701) of *pbp2* was amplified with primers 5'-cgggacccGCGAACTTAGTAAATCGTACTCCTG-3' and 5'-gggctgcagCTGCTGTTGCTGATGTGTC-3'. Underlined lower-case nucleotides represent added *Bam*HI, *Pst*I or *Sac*I restriction enzyme sites required for cloning. PCR was performed with AmpliTaq Gold (Roche), and the products were cloned into *E. coli* INV α F', using the pCR2.1 vector and protocols provided with the Original TA Cloning system (Invitrogen). Plasmid DNA was extracted from selected clones, and sequenced by the University of Toronto Hospital for Sick Children DNA sequencing facility. The 3.2 kb *pfrApbp2* fragments were excised from pCR2.1 with *Bam*HI and *Sac*I, ligated with plasmid pRN5548 (Novick *et al.*, 1993), and electroporated into *S. aureus* RN4220 and RN6390 as detailed in Table 1.

Nucleotide sequence analyses and multilocus sequence typing (MLST). Sequences obtained from this study were compared to *pbp2* from *S. aureus* strains for which genome sequences are available, and other strains for which *pbp2* sequences have been

Table 1. Plasmids and strains used in this study

Plasmid or strain	Description	Source
Plasmids		
pCR2.1	TA cloning vector; Amp ^r	Invitrogen
pCR_AT25	pCR2.1 with 3·1 kb <i>pfrA</i> <i>bp</i> 2 PCR product from <i>S. aureus</i> ATCC 25923	This study
pCR_MS12	pCR2.1 with 3·1 kb <i>pfrA</i> <i>bp</i> 2 PCR product from type A BORSA strain MSH12	This study
pCR_MA15	pCR2.1 with 3·1 kb <i>pfrA</i> <i>bp</i> 2 PCR product from type B BORSA strain MA15	This study
pRN5548	3·1 kb <i>S. aureus</i> plasmid containing pUC18 multiple cloning site; Cm ^r	R. Novick*
pRN_pbAT	3·1 kb insert from pCR_AT25 cloned into <i>Bam</i> HI and <i>Sac</i> I sites of pRN5548	This study
pRN_pbMS	3·1 kb insert from pCR_MS12 cloned into pRN5548	This study
pRN_pbMA	3·1 kb insert from pCR_MA15 cloned into pRN5548	This study
Strains		
<i>E. coli</i>		
INVαF'	Host strain for pCR2.1 and derivatives	Invitrogen
<i>S. aureus</i>		
RN4220	Restriction-deficient laboratory strain; intermediate host for pRN5548 and derivatives	R. Novick
RN6390B	Standard laboratory strain; final host for pRN5548 and derivatives	R. Novick
ATCC 25923	Antibiotic-susceptible test strain	ATCC
ATCC 29213	β-Lactamase-producing oxacillin-susceptible strain	ATCC

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published or deposited in GenBank, as detailed in Table 2. The accession numbers of *pbp2* sequences determined from strains MA15 and MSH12 in this study are AF540019 and AF540020, respectively. Accession numbers AF540021–AF540028 were assigned for the respective partial nucleotide sequences of *pbp2* from additional BORSA isolates MA4, MA6, MA8, MA9, MA13, MA14, MSH7 and OGH2.

MLST was conducted as described for *S. aureus* (Enright *et al.*, 2000) using seven gene loci: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqi*. Each PCR amplicon was sequenced using the forward and reverse primers that were employed for amplification. Automated data analyses and assignment of STs were conducted using the programs provided by the <http://saureus.mlst.net/> site, hosted at Imperial College, London, and funded by the Wellcome Trust.

Table 2. *S. aureus* strains for which PBP2 sequences are known

Strain*	Description	Reference
NCTC 8325 ^a	Standard laboratory strain	Gillaspy <i>et al.</i> (2006)
MSSA 476 ^b	Invasive community-acquired MSSA	Holden <i>et al.</i> (2004)
MW2 ^b	Community-acquired MRSA; related to MW2	Baba <i>et al.</i> (2002)
SRM705 ^b	MSSA	Murakami <i>et al.</i> (1994)
RUSA130 ^a	Derivative of MRSA strain COL	Pinho <i>et al.</i> (1998)
61/5896 ^b	<i>mecA</i> -negative heterogeneous methicillin resistant	Yoshida <i>et al.</i> (2003)
ATCC 6538P ^{b,c,d,e}	MSSA control strain used in BORSA study	Hackbarth <i>et al.</i> (1995)
ATCC 9144 ^{c,d}	MSSA with defect in cell wall synthesis	Fuller <i>et al.</i> (2005)
IL-A ^f	Vancomycin heteroresistant	Boyle-Vavra <i>et al.</i> (2003)
IL-F ^f	Vancomycin heteroresistant	Boyle-Vavra <i>et al.</i> (2003)
N315 ^g	Hospital-acquired MRSA	Kuroda <i>et al.</i> (2001)
Mu50 ^g	Vancomycin intermediate susceptible (VISA)	Kuroda <i>et al.</i> (2001)
MRSA252 ^{b,h}	Epidemic MRSA	Holden <i>et al.</i> (2004)
USA300 ^a	Community-acquired MRSA	Diep <i>et al.</i> (2006)
RF122 ^{b,i}	Bovine isolate; presumed MSSA	L. Herron-Olson and others, GenBank accession no. NC_007622
CDC-6	Clinical isolate with BORSA phenotype	Hackbarth <i>et al.</i> (1995)

*Amino acid substitutions in PBP2: ^aNone, ^bPro₂₈₅→Ala, ^cAla₃₁₅→Glu, ^dSer₅₇₆→Ala, ^eAsp₆₀₆→Ala, ^fSer₇₀₇→Leu, ^gTyr₁₉₇→Cys, ^hThr₁₁₃→Ile, ⁱThr₄₃₉→Val.

RESULTS AND DISCUSSION

Genotypic and phenotypic characterization of BORSA

Genetic diversity among 38 BORSA was assessed by phage typing and PFGE of *Sma*I-digested genomic DNA using established protocols (Simor *et al.*, 2001, 2002). The plasmid profile of each BORSA isolate was also assessed by agarose gel electrophoresis. The details of representative isolates are presented in Table 3. The majority of BORSA ($n=26$) possessed an identical 'type A' PFGE profile of *Sma*I-digested genomic DNA, and the 94/96 phage type (group 5) that was previously noted as a common feature of BORSA (Kernodle *et al.*, 1998). Most type A BORSA ($n=20$) possessed *bla*Z on a 17.2 kb plasmid, as previously noted for BORSA of the 94/96 phage type (Massidda *et al.*, 1996; McMurray *et al.*, 1990). However, six BORSA did not possess a plasmid. Southern hybridization of *Hind*III-digested genomic DNA with a probe specific for *bla*Z revealed that five of these carried a genomic copy of *bla*Z (data not shown), while a single strain MSH12 did not have *bla*Z (Table 3). MLST revealed that MSH12 and two other type A BORSA (MA8 and MA14) were ST25 (Table 3). The 12 BORSA that were not PFGE type A had diverse phage types and eight different PFGE patterns (B–I); they included strains MA15 and MA6, corresponding to ST1 and ST47, respectively, which also did not harbour *bla*Z (Table 3). These two strains, together with MSH12, exhibited MIC values of 1–2 $\mu\text{g ml}^{-1}$ for penicillin, and 4–8 $\mu\text{g ml}^{-1}$ for oxacillin (Table 3). Other isolates that possessed *bla*Z maintained MIC values of 2–8 $\mu\text{g ml}^{-1}$ for oxacillin, whereas the MIC for penicillin was greater than 64 $\mu\text{g ml}^{-1}$ (Table 3).

Based on these data, we conclude that hyperproduction of β -lactamase is not necessary to promote a BORSA phenotype. Although we cannot exclude the possibility of a novel meticillinase that has been described elsewhere (Keseru *et al.*,

2005; Massidda *et al.*, 1992), such an enzyme would have to exhibit little or no activity towards penicillin to account for the low penicillin MIC values that were noted in the few BORSA that lack *bla*Z.

Analysis of amino acid substitutions in PBP2

PBP2 is a 727 amino acid protein, with an N-terminal transglycosylase domain spanning amino acids 76–244, and a C-terminal transpeptidase domain spanning amino acids 360–653 (Fig. 1) that is essential for peptidoglycan cross-linking (Pinho *et al.*, 2001a,b), and is inhibited by the β -lactam family of antibiotics. PBPs also have conserved penicillin-binding motifs (Frere & Joris, 1985; Ghuysen, 1991, 1994), which in PBP2 of *S. aureus* consist of ³⁹⁸SSLK, ⁴⁵⁴SFN and ⁵⁸³KTG (Fig. 1), with Ser³⁹⁸ being the primary active site serine. We anticipated that if variation in PBP2 was responsible for the BORSA phenotype, we would detect amino acid substitutions in the transpeptidase domain of BORSA strains, but not in known PBP2 sequences from MSSA or MRSA.

We first used the BLAST program (www.ncbi.nlm.nih.gov/blast) to assess variation among known PBP2 sequences obtained from *S. aureus* genomes or GenBank depositions, as listed in Table 2. Three strains (COL, USA300 and NCTC 8325-4) had identical sequences, relative to which several others had a limited number of amino acid substitutions, in the N-terminal transglycosylase domain (Thr₁₁₃→Ile; Tyr₁₉₇→Cys), in a spacer segment that separates the transglycosylase and transpeptidase domain (Pro₂₈₅→Ala; Ala₃₁₅→Glu), or close to the C-terminus (Ser₇₀₇→Leu) (Fig. 1, Table 2). Strains ATCC 9144 and ATCC 6538P possessed one or two substitutions in the transpeptidase domain, but are both MSSA (Fuller *et al.*, 2005; Hackbarth *et al.*, 1995). The bovine strain RF122, which also harboured a unique substitution in the transpeptidase domain, is described as being penicillin sensitive (Fitzgerald *et al.*, 2000), and is also assumed to be MSSA. Therefore, among

Table 3. Genotypic and phenotypic traits of BORSA isolates characterized in this study

Strain	Origin*	MIC ($\mu\text{g ml}^{-1}$) for:		<i>bla</i> Z	17.2 kb plasmid	Phage type	PFGE profile	MLST type
		Oxacillin	Penicillin					
MA15	M	4	1	—	—	95	B	ST1
MSH12	T	8	2	—	—	94/96	A	ST25
MA4	M	4	128	+	+	94/96	A	
MA6	M	4	1	—	—	95	F	ST47
MA8	M	4	256	+	+	94/96	A	ST25
MA9	M	4	256	+	+	94/96	A	
MA13	M	8	256	+	+	29; 94/96	A	
MA14	M	8	64	+	(16.7 kb)	94/96	A	ST25
MSH7	T	2	128	+	—	94/96	A	
OGH2	O	4	128	+	+	94/96	A	

*M, Montreal; T, Toronto; O, Ottawa.

Fig. 1. Scale diagram of PBP2, showing the location of the N-terminal transglycosylase domain (stippled) and C-terminal transpeptidase domain (diagonal hatching). The location and sequence of penicillin-binding motifs are indicated by inverted black triangles below the transpeptidase domain. Numbers above PBP2 indicate the location of amino acid substitutions in all known PBP2s, including BORSA strains characterized in this study, relative to PBP2 of *S. aureus* strains ATCC 25923, NCTC 8325 and COL, which harbour identical PBP2 sequences. Two substitutions, Ala₄₅₀→Asp and Ala₄₆₂→Val, which have been reported in PBP2 of strain CDC-6, but for which no sequence accession number was provided, are omitted from this figure.

S. aureus strains that do not exhibit a BORSA phenotype, amino acid substitutions in the transpeptidase domain of PBP2 are unusual, and do not promote resistance.

We then sequenced *pbp2* from the ST1 BORSA strain MA15 and the ST25 BORSA strain MSH12 for comparative purposes. Surprisingly, PBP2 from the ST1 BORSA strain MA15 harboured a single Asp₆₀₆→Val substitution in its transpeptidase domain, at the same site as Asp₆₀₆→Ala in the oxacillin-susceptible strain ATCC 6538P (Table 4). The ST25 BORSA strain MSH12 possessed four substitutions in its transpeptidase domain (Ala₄₀₅→Val; Met₅₈₀→Thr; Gln₆₂₉→Pro; Asn₆₅₀→Asp), all of them different from that of the ST1 strain (Table 4). As the ST1 and ST25 BORSA did not have any common substitutions, we sequenced a *pbp2* segment encoding amino acids 278–701 of PBP2, from seven additional ST25 BORSA and the ST47

strain MA6. Each of the ST25 isolates had the Gln₆₂₉→Pro substitution that was present in MSH12, and previously noted in the prototypic BORSA strain CDC-6 (Hackbarth *et al.*, 1995). This defining feature of ST25 BORSA is the only substitution in strains MA8 and MA9 (Table 4). The other ST25 strains, MSH12, MA4, MA13, MA14, MSH7 and OGH2, harboured three, one, one, four, two and one additional substitutions, respectively, that were unique to each isolate. The ST47 isolate MA6 had a single Thr₅₈₆→Ile substitution in the transpeptidase domain.

This substitution is of interest because it is adjacent to the ₅₈₃KTG penicillin-binding motif, altering the normal ₅₈₃KTGT₅₈₆ sequence to ₅₈₃KTGI, and replaces a small polar amino acid with one with a bulky hydrophobic side chain. When the PBP2 sequence of *S. aureus* is aligned with that of the PBP 2x protein of *Strep. pneumoniae*, the

Table 4. Comparison of amino acid substitutions in the transpeptidase domain of known PBP2 sequences compared to BORSA

Strain	Substitution																	
	V ₃₄₈	S ₃₅₁	A ₄₀₅	G ₄₀₇	Thr ₄₃₉	N ₄₈₅	F ₅₀₃	N ₅₂₃	S ₅₂₆	I ₅₃₈	D ₅₄₁	S ₅₇₆	M ₅₈₀	T ₅₈₆	T ₅₉₃	D ₆₀₆	Q ₆₂₉	N ₆₅₀
RF122					Val													
ATCC 9144												Ala						
ATCC 6538P												Ala				Ala		
MSH12		Ile	Val										Thr				Pro	Asp
MA15	Ala															Val		
MA4										Val							Pro	
MA6														Ile				
MA8																	Pro	
MA9																	Pro	
MA13											Asn						Pro	
MA14						His		Ser							Ser		Pro	Ser
MSH7				Glu			Tyr										Pro	
OGH2									Pro								Pro	

⁵⁸³KTGT sequence in PBP2 is synonymous with the similar ⁵⁴⁷KSGT₅₅₀ penicillin-binding motif in PBP 2x (data not shown). Of particular interest, both laboratory-induced and clinical *Strep. pneumoniae* isolates that exhibit resistance to the extended-spectrum β -lactam antibiotic cefotaxime harbour a Thr₅₅₀→Ala substitution (⁵⁴⁷KSGA₅₅₀) adjacent to this motif (Grebe & Hakenbeck, 1996). Curiously, this substitution promotes cefotaxime resistance, but results in loss of cross-resistance to oxacillin or benzyl penicillin (Grebe & Hakenbeck, 1996). Similarly, it has been noted that a spontaneously selected Pro₄₅₈→Leu substitution adjacent to the ⁴⁵⁴SNF penicillin-binding motif of PBP2 from *S. aureus* promotes reduced affinity for the β -lactam ceftizoxime without affecting the binding of other β -lactam antibiotics (Leski & Tomasz, 2005). Therefore, it is evident that a single amino acid substitution can exert a complex phenotype with respect to susceptibility or resistance towards different β -lactam antibiotics. This may explain why BORSA strains MA15 and MSH12, which lack *blaZ*, exhibit MIC values for oxacillin that are higher than those determined for penicillin.

Expression of BORSA PBP2 confers a borderline-resistant phenotype upon *S. aureus* RN6390

When PCR products containing the *pfrApbp2* genes from the MSSA strain ATCC 25923, and BORSA strains MA15 and MSH12, were cloned into pRN5548 for expression in *S. aureus* RN6390, increased expression of an approximately 81 kDa protein corresponding to the expected size of PBP2 was observed, but not in RN6390 harbouring pRN5548 alone (Fig. 2). Although each RN6390 derivative exhibited increased PBP2 expression, the MIC for oxacillin was not

altered in RN6390 carrying pb_AT derived from the meticillin-sensitive strain ATCC 25923, whereas *S. aureus* RN6390 harbouring pRN_pbMS or pRN_pbMA plasmids had MICs that were comparable to those of the parent BORSA strains MSH12 and MA15, respectively (Table 5). The *pbp2* genes from strains MA15 and MSH12 were selected for expression in *S. aureus* RN6390 because these strains lacked *blaZ*-encoded β -lactamase as a potential contributor to the BORSA phenotype. From these data, we conclude that the BORSA *pbp2* gene alone is sufficient to confer a borderline-resistance phenotype. As the ST1 strain MA15 possessed just one amino acid substitution, Asp₆₀₆→Val, in the transpeptidase domain of its PBP2, and expression of this PBP2 in *S. aureus* RN6390 promoted an oxacillin MIC of 4 $\mu\text{g ml}^{-1}$ (Table 5), our findings strongly implicate a causative role for this substitution in promoting a BORSA phenotype. Although the Gln₆₂₉→Pro substitution appears to be a common feature of all ST25 BORSA isolates, we cannot exclude the possibility that this substitution is a characteristic feature of the ST25 genetic background, irrespective of BORSA phenotype. Future studies will focus on assessing the influence of this and other substitutions that we have identified as specific mediators of a BORSA phenotype.

Analysis of clonal associations among BORSA

Prior to our study, it was known that most BORSA shared a number of common properties, including a high degree of genetic relatedness, the 94/96 phage type, and a 17.2 kb plasmid that encodes *blaZ* (Barg *et al.*, 1991; Massidda *et al.*, 1996; McMurray *et al.*, 1990). Our study has established that these isolates are of the ST25 genetic lineage, but also that the BORSA phenotype is not restricted to this lineage. In this context, our data allow for the first time a comparison of the genetic backgrounds of different BORSA strains in relation to the eight major clonal complexes (CCs) that define the population structure of *S. aureus* (Feil *et al.*, 2003). Strain MA15 was identified as ST1, which is the founding member of the CC1. Other ST1 strains within the CC1 complex include the high-virulence community-acquired MRSA strain MW2, and community-acquired meticillin-susceptible strain MSSA476 (Holden *et al.*, 2004), but these strains

Fig. 2. SDS-PAGE of total cell lysate protein (10 μg) derived from *S. aureus* RN6390 harbouring plasmids pRN_pbMA (lane 1), pRN_pbMS (lane 2), pRN_pbAT (lane 3), pRN5548 (lane 4) or RN6390 alone (lane 5). The black arrow points to a ~82 kDa protein corresponding to the expected size of PBP2, which is expressed at a higher level due to plasmids pRN_pbMA, pRN_pbMS and pRN_pbAT, but not with vector alone (lane 4) or in RN6390 without vector (lane 5).

Table 5. Effect of expressing recombinant PBP2s on MIC values of *S. aureus* RN6390 for penicillin and oxacillin

Strain (plasmid)	MIC ($\mu\text{g ml}^{-1}$) for:	
	Penicillin*	Oxacillin
RN6390	0.023	<0.25
RN6390 (pRN_pbAT)	0.023	0.25
RN6390 (pRN_pbMS)	0.032	4
RN6390 (pRN_pbMA)	0.064	4

*MIC values for penicillin were determined in dilution medium lacking NaCl.

do not have the Asp₆₀₆→Val substitution that we identified in PBP2 of strain MA15. Other ST1 isolates in the *S. aureus* MLST database (<http://saureus.mlst.net/>) include MSSA nasal-carriage isolates, endemic hospital-associated MSSA, and community-acquired MSSA and MRSA, originating from Canada, Denmark, Australia and the USA. As strains of this genetic background have a proven ability to acquire *mecA*-mediated resistance, there is presumably no selection pressure for maintenance of the Asp₆₀₆→Val substitution in the ST1 background.

Similarly, the ST47 group, to which BORSA strain MA6 was assigned, is a single-locus variant of ST45, which defines CC45 (Feil *et al.*, 2003), and includes the Berlin strain of epidemic MRSA (Robinson & Enright, 2004). The nine ST47 isolates in the *S. aureus* MLST database are exclusively meticillin susceptible, but are annotated as the progenitor of the ST45 Berlin strain of epidemic MRSA. Therefore, this genetic background can also readily acquire *mecA*-mediated resistance, which would eliminate any positive selection for maintenance of the single Thr₅₈₆→Ile substitution that we have noted in the transpeptidase domain of this strain.

The ST25 clone to which the majority of BORSA belong is the founding member of CC25. There are presently 34 ST25 isolates in the MLST database; these are exclusively meticillin susceptible, and do not appear to have spawned any successful variants, because reducing the stringency of comparison to find one-, two-, three-, or four-locus variants identifies 17 additional isolates represented by 14 STs. As these STs are not in any of the five major CCs to which MRSA belong (Enright *et al.*, 2002), it appears that members of CC25 cannot readily acquire *mecA*-mediated resistance. For this reason, we propose that there is a positive selection for stable maintenance of the diverse amino acid substitutions that we have noted in nosocomial isolates belonging to the ST25 genetic background. Intriguingly, a recent study that employed MLST to assess genetic variation among 258 bovine-associated *S. aureus* isolates from the USA, Chile and the UK (Smith *et al.*, 2005) revealed that ST25 was one of two predominant STs, ST25 ($n=73$) and ST124 ($n=86$), which collectively formed >60% of bovine isolates. The ST25 strain was the most widely disseminated, being found in the UK, and in 27 of 43 herds that were tested in the USA, and was also significantly more likely to be isolated from teat skin than milk.

Potentially, the prevalence of ST25 *S. aureus* as a bovine-colonizing strain could be related to their propensity to acquire amino acid substitutions in PBP2, and the management of dairy herds (Chriel & Dietz, 2003), in which antibiotics can be administered during 'dry' or non-lactating periods, as a prophylaxis to prevent the incidence of mastitis during future lactation (Bradley & Green, 2004). Such practices could select for ST25 BORSA as a predominant colonizing strain of dairy cattle, and provide a stable reservoir for maintenance of the BORSA phenotype and transmission to humans.

An interesting feature of the ST25 BORSA for which PBP2 sequences were determined is the remarkable variation, apart from Gln₆₂₉→Pro, in the other amino acid substitutions that are present. Studies of *S. aureus* population structure, combined with genome-sequencing projects, strongly support the close association of specific substitutions with strains of a common genetic background. The ST1 strains MW2 and MSSA476 were isolated on different continents (Holden *et al.*, 2004; Lindsay & Holden, 2004), and harbour a common Pro₂₈₅→Ala substitution, while the closely related MRSA strains N315 and Mu50 isolated in Japan in 1982 and 1997, respectively (Enright *et al.*, 2002; Kuroda *et al.*, 2001), share a single Tyr₁₉₇→Cys substitution (Table 2). It is therefore remarkable that most of the ST25 BORSA exhibit unique strain-specific substitutions in their transpeptidase domains (Table 4). The occurrence of eight substitutions in the partial PBP2 sequence of strain MA14 may indicate a high rate of point mutation within the ST25 group. Alternatively, or in addition, these may be compensatory mutations that arise in response to antibiotic exposure.

ACKNOWLEDGEMENTS

J.N. and M.L. were the recipients of scholarship awards from the National Sciences and Engineering Research Council of Canada.

REFERENCES

- Archer, G. L. (1998). *Staphylococcus aureus*: a well-armed pathogen. *Clin Infect Dis* 26, 1179–1181.
- Asahi, Y., Takeuchi, Y. & Ubukata, K. (1999). Diversity of substitutions within or adjacent to conserved amino acid motifs of penicillin-binding protein 2X in cephalosporin-resistant *Streptococcus pneumoniae* isolates. *Antimicrob Agents Chemother* 43, 1252–1255.
- Baba, T., Takeuchi, F., Kuroda, M. & 11 other authors (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 359, 1819–1827.
- Balslev, U., Bremmelgaard, A., Svejgaard, E., Havstrey, J. & Westh, H. (2005). An outbreak of borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) in a dermatological unit. *Microb Drug Resist* 11, 78–81.
- Barg, N., Chambers, H. & Kernodle, D. (1991). Borderline susceptibility to antistaphylococcal penicillins is not conferred exclusively by the hyperproduction of beta-lactamase. *Antimicrob Agents Chemother* 35, 1975–1979.
- Boyle-Vavra, S., Yin, S., Challapalli, M. & Daum, R. S. (2003). Transcriptional induction of the penicillin-binding protein 2 gene in *Staphylococcus aureus* by cell wall-active antibiotics oxacillin and vancomycin. *Antimicrob Agents Chemother* 47, 1028–1036.
- Bradley, A. J. & Green, M. J. (2004). The importance of the nonlactating period in the epidemiology of intramammary infection and strategies for prevention. *Vet Clin North Am Food Anim Pract* 20, 547–568.
- Chriel, M. & Dietz, H. H. (2003). Medication of production animals – cure of malfunctioning animals or production systems? *Acta Vet Scand* 98, 65–70.
- Dessen, A., Mouz, N., Gordon, E., Hopkins, J. & Dideberg, O. (2001). Crystal structure of PBP2x from a highly penicillin-resistant

- Streptococcus pneumoniae* clinical isolate: a mosaic framework containing 83 mutations. *J Biol Chem* **276**, 45106–45112.
- Diep, B. A., Gill, S. R., Chang, R. F. & 9 other authors (2006). Complete genome sequence of USA 300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* **367**, 731–739.
- Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J. & Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* **38**, 1008–1015.
- Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H. & Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci U S A* **99**, 7687–7692.
- Feil, E. J. & Enright, M. C. (2004). Analyses of clonality and the evolution of bacterial pathogens. *Curr Opin Microbiol* **7**, 308–313.
- Feil, E. J., Cooper, J. E., Grundmann, H. & 9 other authors (2003). How clonal is *Staphylococcus aureus*? *J Bacteriol* **185**, 3307–3316.
- Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P. & Spratt, B. G. (2004). eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* **186**, 1518–1530.
- Fitzgerald, J. R., Hartigan, P. J., Meaney, W. J. & Smyth, C. J. (2000). Molecular population and virulence factor analysis of *Staphylococcus aureus* from bovine intramammary infection. *J Appl Microbiol* **88**, 1028–1037.
- Frere, J. M. & Joris, B. (1985). Penicillin-sensitive enzymes in peptidoglycan biosynthesis. *Crit Rev Microbiol* **11**, 299–396.
- Fuller, E., Elmer, C., Nattress, F., Ellis, R., Horne, G., Cook, P. & Fawcett, T. (2005). Beta-lactam resistance in *Staphylococcus aureus* cells that do not require a cell wall for integrity. *Antimicrob Agents Chemother* **49**, 5075–5080.
- Ghuysen, J. M. (1991). Serine beta-lactamases and penicillin-binding proteins. *Annu Rev Microbiol* **45**, 37–67.
- Ghuysen, J. M. (1994). Molecular structures of penicillin-binding proteins and beta-lactamases. *Trends Microbiol* **2**, 372–380.
- Gillaspy, A. F., Worrell, V., Orvis, J., Roe, B. A., Dyer, D. W. & Iandolo, J. J. (2006). The *Staphylococcus aureus* NCTC8325 genome. In *Gram Positive Pathogens*, 2nd edn. Edited by V. Fischetti, R. P. Novick, J. Ferretti, D. Portnoy & J. Rood. Washington, DC: American Society for Microbiology.
- Grebe, T. & Hakenbeck, R. (1996). Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of beta-lactam antibiotics. *Antimicrob Agents Chemother* **40**, 829–834.
- Hackbarth, C. J., Kocagoz, T., Kocagoz, S. & Chambers, H. F. (1995). Point mutations in *Staphylococcus aureus* PBP 2 gene affect penicillin-binding kinetics and are associated with resistance. *Antimicrob Agents Chemother* **39**, 103–106.
- Hartman, B. J. & Tomasz, A. (1984). Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J Bacteriol* **158**, 513–516.
- Hiramatsu, K., Cui, L., Kuroda, M. & Ito, T. (2001). The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol* **9**, 486–493.
- Holden, M. T., Feil, E. J., Lindsay, J. A. & 42 other authors (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* **101**, 9786–9791.
- Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., Tienasitorn, C. & Hiramatsu, K. (2001). Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* **45**, 1323–1336.
- Kernodle, D. S., Classen, D. C., Stratton, C. W. & Kaiser, A. B. (1998). Association of borderline oxacillin-susceptible strains of *Staphylococcus aureus* with surgical wound infections. *J Clin Microbiol* **36**, 219–222.
- Keseru, J. S., Gal, Z., Barabas, G., Benko, I. & Szabo, I. (2005). Investigation of beta-lactamases in clinical isolates of *Staphylococcus aureus* for further explanation of borderline methicillin-resistance. *Chemotherapy* **51**, 300–304.
- Kuroda, M., Ohta, T., Uchiyama, I. & 34 other authors (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225–1240.
- Laible, G., Spratt, B. G. & Hakenbeck, R. (1991). Interspecies recombinational events during the evolution of altered PBP 2x genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Mol Microbiol* **5**, 1993–2002.
- Leski, T. A. & Tomasz, A. (2005). Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. *J Bacteriol* **187**, 1815–1824.
- Lindsay, J. A. & Holden, M. T. (2004). *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol* **12**, 378–385.
- Louie, L., Matsumura, S. O., Choi, E., Louie, M. & Simor, A. E. (2000). Evaluation of three rapid methods for detection of methicillin resistance in *Staphylococcus aureus*. *J Clin Microbiol* **38**, 2170–2173.
- Louie, L., Goodfellow, J., Mathieu, P., Glatt, A., Louie, M. & Simor, A. E. (2002). Rapid detection of methicillin-resistant staphylococci from blood culture bottles by using a multiplex PCR assay. *J Clin Microbiol* **40**, 2786–2790.
- Massanari, R. M., Pfaller, M. A., Wakefield, D. S., Hammons, G. T., McNutt, L. A., Woolson, R. F. & Helms, C. M. (1988). Implications of acquired oxacillin resistance in the management and control of *Staphylococcus aureus* infections. *J Infect Dis* **158**, 702–709.
- Massidda, O., Montanari, M. P. & Valardo, P. E. (1992). Evidence for a methicillin-hydrolysing beta-lactamase in *Staphylococcus aureus* strains with borderline susceptibility to this drug. *FEMS Microbiol Lett* **71**, 223–227.
- Massidda, O., Montanari, M. P., Mingoia, M. & Valardo, P. E. (1996). Borderline methicillin-susceptible *Staphylococcus aureus* strains have more in common than reduced susceptibility to penicillinase-resistant penicillins. *Antimicrob Agents Chemother* **40**, 2769–2774.
- McDougal, L. K. & Thornsberry, C. (1986). The role of beta-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalosporins. *J Clin Microbiol* **23**, 832–839.
- McMurray, L. W., Kernodle, D. S. & Barg, N. L. (1990). Characterization of a widespread strain of methicillin-susceptible *Staphylococcus aureus* associated with nosocomial infections. *J Infect Dis* **162**, 759–762.
- Mouz, N., Di Guilmi, A. M., Gordon, E., Hakenbeck, R., Dideberg, O. & Vernet, T. (1999). Mutations in the active site of penicillin-binding protein PBP2x from *Streptococcus pneumoniae*. Role in the specificity for beta-lactam antibiotics. *J Biol Chem* **274**, 19175–19180.
- Murakami, K., Fujimura, T. & Doi, M. (1994). Nucleotide sequence of the structural gene for the penicillin-binding protein 2 of *Staphylococcus aureus* and the presence of a homologous gene in other staphylococci. *FEMS Microbiol Lett* **117**, 131–136.
- National Committee for Clinical Laboratory Standards (2003). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard*, 6th edn. NCCLS

- document M7-A6. Wayne PA: National Committee for Clinical Laboratory Standards.
- Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B. & Moghazeh, S. (1993).** Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J* **12**, 3967–3975.
- Pinho, M. G., de Lencastre, H. & Tomasz, A. (1998).** Transcriptional analysis of the *Staphylococcus aureus* penicillin-binding protein 2 gene. *J Bacteriol* **180**, 6077–6081.
- Pinho, M. G., de Lencastre, H. & Tomasz, A. (2001a).** An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. *Proc Natl Acad Sci U S A* **98**, 10886–10891.
- Pinho, M. G., Filipe, S. R., de Lencastre, H. & Tomasz, A. (2001b).** Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*. *J Bacteriol* **183**, 6525–6531.
- Rice, K., Huesca, M., Vaz, D. & McGavin, M. J. (2001).** Variance in fibronectin binding and *fnb* locus polymorphisms in *Staphylococcus aureus*: identification of antigenic variation in a fibronectin binding protein adhesin of the epidemic CMRSA-1 strain of methicillin-resistant *S. aureus*. *Infect Immun* **69**, 3791–3799.
- Robinson, D. A. & Enright, M. C. (2004).** Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* **10**, 92–97.
- Simor, A. E., Ofner-Agostini, M., Bryce, E., Green, K., McGeer, A., Mulvey, M. & Paton, S. (2001).** The evolution of methicillin-resistant *Staphylococcus aureus* in Canadian hospitals: 5 years of national surveillance. *CMAJ* **165**, 21–26.
- Simor, A. E., Ofner-Agostini, M., Bryce, E., McGeer, A., Paton, S. & Mulvey, M. R. (2002).** Laboratory characterization of methicillin-resistant *Staphylococcus aureus* in Canadian hospitals: results of 5 years of National Surveillance, 1995–1999. *J Infect Dis* **186**, 652–660.
- Smith, E. M., Green, L. E., Medley, G. F. & 7 other authors (2005).** Multilocus sequence typing of intercontinental bovine *Staphylococcus aureus* isolates. *J Clin Microbiol* **43**, 4737–4743.
- Suggs, A. H., Maranan, M. C., Boyle-Vavra, S. & Daum, R. S. (1999).** Methicillin-resistant and borderline methicillin-resistant asymptomatic *Staphylococcus aureus* colonization in children without identifiable risk factors. *Pediatr Infect Dis J* **18**, 410–414.
- Tortosa, P. & Dubnau, D. (1999).** Competence for transformation: a matter of taste. *Curr Opin Microbiol* **2**, 588–592.
- van Griethuysen, A., Bes, M., Etienne, J., Zbinden, R. & Kluytmans, J. (2001).** International multicenter evaluation of latex agglutination tests for identification of *Staphylococcus aureus*. *J Clin Microbiol* **39**, 86–89.
- Valardo, P. E. (1993).** The ‘borderline methicillin-susceptible’ *Staphylococcus aureus*. *J Antimicrob Chemother* **31**, 1–4.
- Valardo, P. E., Montanari, M. P., Biavasco, F., Manso, E., Ripa, S. & Santacroce, F. (1993).** Survey of clinical isolates of *Staphylococcus aureus* for borderline susceptibility to antistaphylococcal penicillins. *Eur J Clin Microbiol Infect Dis* **12**, 677–682.
- Yamazumi, T., Marshall, S. A., Wilke, W. W., Diekema, D. J., Pfaller, M. A. & Jones, R. N. (2001).** Comparison of the Vitek Gram-Positive Susceptibility 106 card and the MRSA-screen latex agglutination test for determining oxacillin resistance in clinical bloodstream isolates of *Staphylococcus aureus*. *J Clin Microbiol* **39**, 53–56.
- Yoshida, R., Kuwahara-Arai, K., Baba, T., Cui, L., Richardson, J. F. & Hiramatsu, K. (2003).** Physiological and molecular analysis of a *mecA*-negative *Staphylococcus aureus* clinical strain that expresses heterogeneous methicillin resistance. *J Antimicrob Chemother* **51**, 247–255.