**Stenotrophomonas maltophilia** strains isolated from a university hospital in Japan: genomic variability and antibiotic resistance

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The diversity within the genetic and antibiotic resistance profiles and the production of virulence-associated enzymic activities of 66 *Stenotrophomonas maltophilia* strains collected from a university hospital in Japan in 2005 were studied. PFGE analysis of the collection indicated that a variety of profiles were present. MLST analysis of nine selected strains showed that four of the six sequence types identified were novel. These results indicated that there was a high degree of genetic diversity between the strains and that *S. maltophilia* strains isolated in Japan might be genetically divergent from those in Europe. The majority of strains were resistant to piperacillin (93.9%), ceftazidime (84.8%), imipenem (100%), aztreonam (98.5%), gentamicin (81.8%), amikacin (87.9%), ciprofloxacin (84.8%), tetracycline (97.0%), and chloramphenicol (78.8%), although levofloxacin was effective against 77.3% of the strains. Most of the strains showed multidrug resistance and carried the class 1 integron, but no strain showed transmission of antibiotic resistance by conjugation. Although haemolytic activity was not detected in any of the strains, protease and lipase activities were detected in 86.4% and 31.8% of the strains, respectively.

**INTRODUCTION**

*Stenotrophomonas maltophilia* is a Gram-negative bacterium that can grow in most humid environments, and can cause serious nosocomial infections, although it is not highly virulent (Looney *et al.*, 2009). It has emerged as an important opportunistic pathogen, and the frequency of isolation of this bacterium as a cause of serious infection is reported to be increasing (Liau *et al.*, 2002; Chang *et al.*, 2004; Tan *et al.*, 2008). In many cases, treatment of *S. maltophilia* infection is problematic because of its high-level resistance to multiple classes of antibiotics (Alonso & Martinez, 1997; Nicodemo & Paez, 2007). This bacterium carries a large number of antibiotic resistance determinants, including antibiotic-inactivating enzymes and efflux pumps, such as SmeABC, SmeDEF, SmeJKL and SmeYZ (Chang *et al.*, 2004; Okazaki & Avison, 2007; Crossman *et al.*, 2008; Okazaki & Avison, 2008). In particular, the chromosomally encoded β-lactamases L1 and L2 confer resistance to β-lactams (Okazaki & Avison, 2008). Epidemiological studies of clinical *S. maltophilia* isolates have shown genetic diversity, probably as a result of the selection of naturally present *S. maltophilia* from among other bacteria by antibiotic pressure (Hauben *et al.*, 1999; Valdezate *et al.*, 2004). However, differentiation between contamination and infection is difficult, and transmissions between patients and healthcare workers have been reported (García de Viedma *et al.*, 1999). For this reason, it is important to identify antibiotic resistance patterns and types of *S. maltophilia* isolates in the context of hospital infection control. Some molecular-typing studies have suggested that certain strains of *S. maltophilia* have favourable characteristics for infection and colonization (Coenye *et al.*, 2004; Gould *et al.*, 2006). As in other countries, this bacterium is problematic in Japan and the frequency of its isolation in clinical settings is increasing (Araoka *et al.*, 2010).

The present study describes the investigation of *S. maltophilia* isolates obtained from a university hospital in Japan in 2005. The study investigated antibiotic resistance, PFGE and MLST profiles and the production of virulence-related enzymes in the isolates.

**METHODS**

**Bacterial isolates.** A total of 66 *S. maltophilia* isolates were obtained from a variety of specimens obtained from Gunma University Hospital in 2005. Only one isolate was obtained from each patient.

**Multilocus sequence typing (MLST) analysis.** MLST was performed according to Kaiser *et al.* (2009) and the protocol on the website of the *S. maltophilia* MLST database (http://pubmlst.org/...
smaltophilia/). Loci were amplified using a Veriti 96-well Thermal Cycler (Applied Biosystems) and the rTaq PCR enzyme (Take Bio). PCR product was treated with ExoSAP-IT (USB) before sequencing with a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) on an ABI Prism 310 Sequencer (Applied Biosystems). Sequence data were processed with GENETYX v8.0.

Pulsed-field gel electrophoresis (PFGE) analysis. Agarose gel plugs containing chromosomal DNA were digested overnight at 37 °C using the restriction enzyme XbaI (Roche Diagnostics). PFGE was performed using a CHEF-MAPPER according to the manufacturer’s protocol (Bio-Rad). Phylogenetic analysis based on the PFGE profiles was performed with the FPQuest Software (Bio-Rad).

Haemolytic activity test. Bacterial colonies purified by single colony isolation were streaked on 5 % sheep blood agar plates (Nissui Pharmacy) and incubated overnight at 37 °C. A colony with a haemolytic zone was classed as a haemolysin-positive strain.

Protease activity test. Casein hydrolysis was tested by streaking singly purified colonies onto Mueller–Hinton (MHI) agar plates (Difco) containing 3 % skimmed milk (Wako Pure Chemicals). The presence of a transparent zone around the colony after incubation at 37 °C for 48 h indicated a positive result.

Lipase activity test. Tryptic soya agar plates (Difco) containing 1 % Tween 80 (Wako Pure Chemicals) were used to test for lipase activity. Singly purified colonies were streaked on the plates, and incubated at 37 °C for 24 h. The presence of a turbid halo around the colony indicated a positive result.

Susceptibility test. The MIC for each antibiotic was determined according to the criteria of the National Committee for Clinical Laboratory Standards using MH agar (CLSI, 2007). Overnight cultures of the strains grown in MH broth were diluted 100-fold with fresh broth. One loopful (5 μl: about 5 × 10^6 to 10^7 cells) of each dilution was transferred to the agar plates containing the relevant antibiotics.

Detection of integrons. Class 1, class 2 and class 3 integrons were detected by PCR using the rTaq polymerase and sets of specific primers for class 1 integrase (int1L, 5'-ACATGTGATGGCGACGG-CAAGA-3'; int1R, 5'-ATTTCGTCCTGCCTGGGCGG-3'), class 2 integrase (int2L, 5'-CAGCGATATGCGACAAAAAGGT-3'; int2R, 5'-GTAGCAAACGAGTGACGAAATG-3') and class 3 integrase (int3L, 5'-GCTCCGGCGACGGCCTTGTACT-3') and int3R, 5'-ACGGATCTGCC-AACCAGTACT-3') (Ploy et al., 2000). Chromosomal DNA was isolated from each isolate using ISOLPLANT (Wako Pure Chemicals) according to the manufacturer’s instructions for use as a template.

Conjugal transfer of antibiotic resistance. An overnight culture of each strain grown in LB broth was mixed with the recipient strain, ATCC 17666rif or ATCC 13037rif, which are rifampicin-resistant strains derived by spontaneous mutation from S. maltophilia ATCC 17666 and S. maltophilia ATCC 13037, respectively. The ratio of donor to recipient was 1:10. Five microlitres of the mixture was spotted onto an L agar plate, and incubated at 37 °C for 8 h. After incubation, bacteria were scraped off the agar plate and suspended in 1 ml saline. One hundred microlitres of the suspension was plated on a selective agar plate containing the appropriate antibiotics to select transconjugants. Plates were incubated overnight at 37 °C. The concentrations of antibiotics used in the mating experiments were as follows: ciprofloxacin, 20 μg ml⁻¹; chloramphenicol, 20 μg ml⁻¹; gentamicin, 20 μg ml⁻¹; kanamycin, 40 μg ml⁻¹; rifampicin, 40 μg ml⁻¹.

RESULTS

PFGE profile analysis

PFGE analysis was used to determine the genetic similarity among the 66 clinical isolates of S. maltophilia. As shown in Fig. 1, most of the strains did not show any significant level of relatedness, although these strains were isolated from the same hospital.

MLST analysis

The results of the PFGE analysis were used to select nine strains that did not show any close relationship with each other for further analysis by MLST. Table 1 shows the results of the MLST analysis. Of the six sequence types (STs) that were identified, ST23 and ST27 have been reported previously. Isolates SmGM5 and SmGM47 belonged to ST27, and SmGM55 belonged to ST23. The other six isolates belonged to four novel sequence types (STs 83–86) that were identified for the first time in this study.

Antimicrobial susceptibility

The results of the susceptibility testing (Table 2) showed that most of the S. maltophilia isolates were resistant to piperacillin (93.9 %), ceftazidime (84.8 %), imipenem (100 %), aztreonam (98.5 %), gentamicin (81.8 %), amikacin (87.9 %), ciprofloxacin (84.8 %), tetracycline (97.0 %) and chloramphenicol (78.8 %). Among the antibiotics tested, levofloxacin was effective against 73.3 % of the isolates. Most of the isolates (81.8 %) were resistant to multiple antibiotics (six or more of the ten drugs tested), and 9.1 % (six isolates) were resistant to all antibiotics tested.

Conjugal transfer of antibiotic resistance

The transferability of antibiotic resistance was examined with the exception of β-lactam resistance, as S. maltophilia is intrinsically resistant to all β-lactams because it carries two chromosomally encoded β-lactamases. No antibiotic resistance was transferred, indicating that resistance determinants were not carried on a conjugative plasmid or integrative and conjugative element, which would have been detected in the mating conditions used in this experiment.

Detection of integrons by PCR

The presence of an integron was examined by PCR with specific primers sets for the integrase of class 1, 2 and 3 integrons. PCR products were detected with the class 1 integron specific primers from 30 of the 66 strains (45.5 %). In contrast, no PCR product was detected with class 2 and 3 specific primers, indicating that those isolates did not carry class 2 or 3 integrons.
Enzymic activities

Although no strain showed haemolytic activity on sheep blood agar plates, indicating that they did not produce haemolysin, 57 strains (86.4%) produced a transparent zone around the colony on skimmed milk plates, indicating that they produced protease. Twenty-one strains (31.8%) produced a turbid halo around the colony on tryptic soy agar plates, and were confirmed to be lipase-positive. Eighteen (85.7%) of the 21 lipase-positive strains also produced protease.

DISCUSSION

Although the 66 strains examined in this study were isolated from the same university hospital in 2005, they did not show any significant similarities in PFGE analysis. These results indicated that there was a high degree of genetic diversity among the 66 clinical isolates and that cross-transmission among patients, or infection from a common source, did not occur. Previous studies using a variety of typing approaches have also shown, with the exception of rare outbreaks, that this species has a high genodiversity even when strains are isolated in the same hospital (Hauben et al., 1999; Valdezate et al., 2004). These results strongly suggested that those strains were not transferred within the hospital, but brought into the
Antibiotic susceptibility results for the clinical isolates of *S. maltophilia*.

<table>
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<tr>
<th>Antibiotic agent</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (µg ml&lt;sup&gt;-&lt;/sup&gt;)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg ml&lt;sup&gt;-&lt;/sup&gt;)</th>
<th>Range</th>
<th>Break point&lt;sup&gt;a&lt;/sup&gt; (µg ml&lt;sup&gt;-&lt;/sup&gt;)</th>
<th>Percentage of susceptible strains</th>
<th>Percentage of resistant strains</th>
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*Break points for CAZ and LVFX are adopted from the CLSI Manual (CLSI, 2007) and the other break points are taken from those for *P. aeruginosa* listed in the CLSI Manual.*

It has been shown that integrons in Gram-negative isolates from clinical settings are associated with decreased susceptibility to multiple antibiotics. In this study, we examined the presence of integrons using PCR and specific primers, and detected class 1 integrons in 30 isolates (45.5%), indicating that the integron plays a major role in the acquisition of multiple antibiotic resistances in *S. maltophilia* as it does in other bacteria (Ploy et al., 2000; Liaw et al., 2010). However, it remains unclear how the class 1 integron spread among these isolates, because it has been spread by efficient genetic exchange system(s) a long time ago.
an efficient genetic exchange system has not yet been found.

Since the first report describing the isolation of *S. maltophilia*, the frequency of isolation has continued to rise, and it has become a cause of nosocomial infection due to its innate resistance to antibiotics. Despite its high incidence, its virulence factors have been poorly characterized (Looney et al., 2009). In this study, we characterized 66 isolates with regard to the production of three virulence factors, haemolysin, protease and lipase. Protease activity was detected in most of the isolates (86.4 %), lipase production was observed at a much lower incidence (31.8 %) and haemolysin production was not observed at all. Although most of lipase-positive isolates (85.7 %) showed protease activity, those traits did not seem to be essential factors for the spread of this bacterium in hospitals. It is well known that *S. maltophilia* does not carry strong virulence factors (Looney et al., 2009). The results obtained in this study indicate that, as in other countries, *S. maltophilia* is mainly acquired in the community and carried over into hospitals in Japan, where it may be isolated from a hospital specimen. Therefore, it is difficult to differentiate between colonization or contamination, or both, and true infection by *S. maltophilia* within the clinical setting.

*S. maltophilia* causes serious infection in immunocompromised patients and its isolation rate as a cause of infection is reported to be on the increase, despite the uncertainty of the source or origin and the lack of strong virulence factors. *S. maltophilia* could become a greater concern in nosocomial infection and it is essential to know more about this bacterium. To gain better understanding of this bacterium and its role in infection, it is necessary to accumulate epidemiological data from *S. maltophilia* clinical isolates, and examine the expression of the putative virulence genes predicted from the *S. maltophilia* genome sequence, because some strains are reported to carry characteristics that favour colonization and infection.

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**REFERENCES**


