Distribution and diversity of a cadmium resistance (\textit{cadA}) determinant and occurrence of IS257 insertion sequences in Staphylococcal bacteria isolated from a contaminated estuary (Seine, France)

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Abstract

Water and sediments of the Seine estuary are contaminated by chemicals, especially cadmium, which favors survival and growth of cadmium-resistant bacteria. We investigated the diversity of the \textit{cadA} gene, which encodes a Cd$^{2+}$/ATPase protein transporter, in the microbial community of the Seine estuary. The \textit{cadA} gene first isolated from \textit{S. aureus} (pI258) was prevalent, with a conservation better than 98% identity, despite its presence in host bacteria of diverse species and genera. We report for the first time, eleven distinct \textit{Staphylococcus} species, and also bacteria belonging to the \textit{Micrococcus} and \textit{Halobacillus} genera carrying the \textit{cadA} gene. This \textit{cadA} determinant was mostly plasmid-borne in the \textit{Staphylococcus} genus, and IS257 sequences, which are known to participate in antibiotic resistance gene dissemination in \textit{S. aureus}, were found to be located near to the \textit{cadA} gene in 16/31 cadmium-resistant \textit{Staphylococcus} strains and one \textit{Micrococcus} strain. This suggests that IS257 has also contributed to the dissemination of the \textit{cadA} resistance gene among staphylococci. These findings also emphasize on the existence of Staphylococcal bacteria in contaminated natural niches outside hospital environments.

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1. Introduction

Bacteria of the genus \textit{Staphylococcus} are part of the commensal flora of mammals and birds [1,2]. The \textit{Staphylococcus} species that are pathogenic to humans, \textit{Staphylococcus aureus} and \textit{Staphylococcus haemolyticus}, are often isolated from hospital environments where they rapidly adapt to commonly used antibiotics and antiseptics [3–7]. The corresponding antibiotic resistance genes are often associated with metal resistances, especially resistance to cadmium [8–13]. The natural resistance of these bacteria to environmental factors (halotolerance, desiccation) allows them to persist outside their hosts (dust, soil, water or other niches [14]), but there have been very few studies investigating this survival behavior.

The cadmium resistance of \textit{S. aureus} is mediated by several genetic determinants [11,15]. One, the \textit{cadCA} operon, has been studied extensively [16–21]. The \textit{cadA} gene encodes resistance to Cd$^{2+}$ and Zn$^{2+}$ [11,22] by an energy-dependent efflux mechanism [16]. The CadA protein is a Cd$^{2+}$/ATPase transporter, containing the eight transmembrane domains characteristic of P-class ATPases [10,21]. In \textit{S. aureus}, \textit{cadA} is located on large plasmids, such as pI258 [17,19] or pXU5 [12], and on the chromosome (Tn554) [13,23]. Homologs of \textit{cadA} have only been described in three other Gram-positive species: \textit{Lactococcus lactis}, \textit{Listeria monocytogenes} (Tn5422) [21,24] and \textit{Bacillus firmus} [25]. These \textit{cadA} homologs are carried by plas-
mids in \textit{L. lactis} and \textit{L. monocytogenes} and on the chromosome in \textit{B. firmus}. More recently, the \textit{cadA} gene has also been found on the chromosome of the Gram-negative species, \textit{Stenotrophomonas maltophilia}, upstream of an IS257 insertion sequence [26]. The flanking insertion sequences and the unusual G+C content of the locus suggested lateral transfer of the gene from a Gram-positive to this Gram-negative bacteria [26].

The IS257 insertion sequence is a member of the IS6 family [27]. In \textit{S. aureus}, IS257 elements are often associated with trimethoprim, mercury, methicillin and tetracycline resistance determinants [28]. Copies of IS257 often flank such resistance genes in an organization reminiscent of a composite transposon [6], suggesting that they contribute to the appearance of multiresistant \textit{S. aureus} strains [29].

Antibiotic and heavy metal resistance genes are often plasmid-borne in \textit{Staphylococcus} bacteria. The plasmids can be divided into four classes on the basis of their sizes and the occurrence of transposable elements [6,9]: (i) 5 kb, such as \textit{pT181}, \textit{pC194}, \textit{pSN2} and \textit{pE194}, these plasmids rarely carry insertion sequences, probably due to a size constraint imposed by their replication strategy, (ii) 8 to 13 kb, such as \textit{pSK639}, these plasmids carry two or three copies of IS257, (iii) 15 to 40-kb non-conjugative plasmids, they encode many resistance genes associated with transposable elements, they include members of the \textit{pSK1} family and multiresistance, \textit{β}-lactamase and heavy metal resistance plasmids such as \textit{pI258}, \textit{pI147}, \textit{pI524} and \textit{pXU5}, which carry the \textit{cadA} gene, and (iv) conjugationally proficient plasmids of over 40 kb, such as \textit{pSK41}, \textit{pGO1} and \textit{pJE1}, most of them harbor many copies of IS257. They can mobilize other plasmids and favor the horizontal transfer of non-conjugative plasmids or chromosomal segments by cointegration [6,7,9].

Metal resistance genes are common in microbial communities growing in contaminated environments. Heavy metals exert a strong selective pressure on microorganisms, resulting in major changes in the structure and diversity of the microbial community [30–34]. The species composition of bacterial communities is affected by contamination: both pre-existing resistant bacteria take up a larger proportion of the population and new resistant microorganisms are selected. This phenomenon is generally accompanied by bacterial gene transfers [34–38].

The water and sediment of the Seine estuary (France) have become contaminated with heavy metals, especially cadmium [39]. Exposure to a toxic concentration of dissolved cadmium is accompanied by an increase in the \textit{cadA} gene level in the total DNA of the microbial community [40]. In the present study, we analyzed the diversity of the \textit{cadA} gene and its host bacteria, focussing on cadmium-resistant bacteria and especially \textit{staphylococci}, from the Seine estuary. In this way, the potential role of IS257 in \textit{cadA} gene dissemination among these bacteria has also been evaluated.

2. Materials and methods

2.1. Sampling

Sampling sites in the Seine estuary (France) were (i) wastewater from treated effluent which is the major source of the fecal contamination in this downstream estuary (two sampling series), (ii) water at the mouth of the river where salinity of the estuary water is maximal (three sampling series), (iii) Northern intertidal mudflats, where the fine particles from further upstream with their associated contaminants accumulate (one sampling series) and (iv) mussels filtering the suspended matter at the mouth of the river (one sampling series). Water samples were collected in Niskin bottles, transferred into sterile bottles (1 l) and conserved at 4°C until microbiological analysis (within 8 h) or into sterile centrifugation tubes containing SDS (final concentration, 0.1%) and \textit{Na₂EDTA} (1 mM, pH 8.0) for nucleic acid analysis [41]. Sediment samples from the northern intertidal mudflats (25 g wet weight) were collected in sterile polypropylene tubes (Corning), and stored at 12°C until microbiological analysis (within 8 h) or at −18°C until nucleic acid analysis. Mussels were collected and stored at 4°C until analysis.

2.2. Isolation and identification of bacterial strains

\textit{Staphylococcus} strains were isolated from water and sediment samples after incubation on Chapman medium (AES Laboratory) supplemented with 500 μM cadmium for 24 to 48 h at 30°C. These growth conditions favor \textit{Staphylococcus} but also halotolerant strains of the \textit{Halobacillus} and \textit{Micrococcus} genera. This temperature was chosen to avoid curing of the staphylococcal plasmids [9]. \textit{Staphylococcus} and \textit{Micrococcus} were identified phenotypically by the miniaturized biochemical ApiStaph method (BioMérieux). The identity of \textit{S. aureus} isolates was confirmed by the coagulase activity assay (‘staphyslide test, BioMérieux). \textit{S. aureus} was isolated from mussels as described in the AFNOR norm (NF V08-014). \textit{Bacillus} and \textit{Halobacillus} were identified genotypically by sequencing 1003 bp of their 16S rDNA. These sequences were analyzed using the BlastN programs available at http://www.ncbi.nlm.nih.gov/BLAST. Staphylococcal plasmid curing was performed as described by Novick [42].

2.3. Extraction of nucleic acids from estuary water and sediments

Nucleic acids were extracted from water samples as previously described [41]. The Fast DNA Spin kit and the FastPrep FP120 cell disputer (Bio-101, CA, USA) were used to extract nucleic acids from 0.5 g (wet weight) of sediment [43]. Crude DNA was purified from water and sediment by elution through an Elutip-d column (Shleicher and Schuell, Germany), followed by precipitation [44].
DNA concentrations were estimated by visualizing samples spotted on ethidium bromide agarose plates under UV light [44].

2.4. Extraction of chromosomal and plasmid DNA

Plasmid DNA was extracted from Gram-negative bacteria using the Plasmid Midi purification kit according to the manufacturer’s recommendations (Qiagen). Plasmid DNA was similarly extracted from Gram-positive strains with the following modifications. Bacteria were grown overnight at 30°C with agitation. After centrifugation the following modifications. Bacteria were grown DNA was similarly extracted from Gram-positive strains the manufacturer’s recommendations (Qiagen). Plasmid DNA was similarly extracted from Gram-positive strains using the Plasmid Midi purification kit according to the manufacturer’s recommendations (Qiagen). Plasmid DNA was similarly extracted from Gram-positive strains using the Plasmid Midi purification kit according to the manufacturer’s recommendations (Qiagen). Plasmid DNA was similarly extracted from Gram-positive strains using the Plasmid Midi purification kit according to the manufacturer’s recommendations (Qiagen).

2.5. Screening plasmids and bacteria for the cadA gene

Bacterial colonies and plasmids were screened for the cadA gene by DNA hybridization and Southern blot analysis, respectively [44]. The cadA probe was prepared from pKPY11, a recombinant plasmid that contains the entire cadA operon from pT7-5 [21]. The probe, a 1058-bp DNA fragment, was labeled with digoxigenin-dUTP using cad1 and cad2 primers, as previously described [40] (Boehringer, Mannheim, Germany).

2.6. Primer design and PCR amplification

To amplify IS257 insertion sequences, the OG1 primer ([GTTTCTGTTGCAAAATWR]-3') was designed to anneal to the first 18 bp of the IS257 inverted repeat sequences from IS257-1, IS257-2, IS257-3, IS257R1 and IS257R2 (http://www.is.biotoul.fr) [27]. The inv-OG1 primer ([YWACTTGTGCAAAGAACC]-3') was the inverse sequence of OG1. The region between IS257 and the cadA gene was amplified using invOG1 and cad1 or invOG1 and cad2.

The cadA gene was amplified using cad1 and cad2 [40]. The eubacterial 16S ribosomal DNA (rDNA) sequence was amplified using primers 338F ([GTTTCTGTTGCAAAATWR]-3') and 1404R ([GGCTTGTGTAACGCGGCAAGC]-3') as recommended by the supplier (Eurogentec). The generated fragments were subjected to electrophoresis.

2.7. Cloning and sequencing of PCR products

The PCR products were cloned with the pGEM-Teasy vector systems kit, as described by the manufacturer (Promega). The DNA sequences of both strands were determined by Genome Express (Grenoble, France). The DNA and deduced amino acid sequences were analyzed using the BlastN and BlastP programs available at http://www.ncbi.nlm.nih.gov/BLAST. The DNA sequences reported in this paper have been deposited in the GenBank databases under the following accession numbers: AY135717 to AY135727.

2.8. RFLP analysis

The cadA PCR product (400 ng) was digested with RsaI in a 20-μl volume as recommended by the supplier (Eurogentec). The generated fragments were subjected to electrophoresis.

2.9. Molecular phylogenetic analysis

A multiple alignment of CadA protein sequences (320 aa) was constructed using the ClustalW program, version 1.74 [46]. The neighbor-joining and maximum-parsimony methods were used to construct phylogenetic trees using the Phylip program version 3.5 [47]. A bootstrap test [48] was constructed with 1000 resamplings. Trees were visualized with the Treeview program [49].

3. Results

3.1. Cadmium-resistant bacteria isolated from the Seine estuary

Bacteria resistant to 500 μM cadmium were isolated from water and sediment samples collected from four distinct sites in the Seine estuary (Fig. 1). Although the growth conditions favored isolation of cadmium-resistant Staphylococcus, they also allowed the isolation of other halotolerant aerobic bacteria naturally present in the Seine.
estuary, including *Micrococcus*, *Bacillus* and *Halobacillus* strains. Among these CdR bacteria, those hybridizing with the cadA gene from *S. aureus* [21] were identified and studied further. Of the 37 bacteria that hybridized with the cadA probe, 31 belonged to the *Staphylococcus* genus (12 different species), two to the *Micrococcus* genus, three to the *Bacillus* genus and one to the *Halobacillus* genus (Table 1). The isolates belonging to the same species were different strains, as shown by their different antibiotic resistance profiles (data not shown). During the study, two of the *Bacillus* strains (MSA 233 and MSA 236) became 'unculturable'. Although no bacterial strain harboring the cadA gene has previously been described in an aquatic environment, one *Halobacillus trueperi* strain, six *Staphylococcus* and one *Bacillus* species were isolated from treated wastewater effluent, and seven *Staphylococcus* and one *Bacillus* species were isolated from estuary water (Table 1). The isolates belonging to the same species were different strains, as shown by their different antibiotic resistance profiles (data not shown). During the study, two of the *Bacillus* strains (MSA 233 and MSA 236) became ‘unculturable’.

Although no bacterial strain harboring the cadA gene has previously been described in an aquatic environment, one *Halobacillus trueperi* strain, six *Staphylococcus* and one *Bacillus* species were isolated from treated wastewater effluent, and seven *Staphylococcus* and one *Bacillus* species were isolated from estuary water (Table 1). Only one *Staphylococcus* and one *Bacillus* species were isolated from the mudflats. Although many *S. aureus* isolates were collected from mussels, no *S. aureus* isolate could be obtained from the total of 300 bacteria isolated from three independent series of mudflats samples (data not shown).

### 3.2. Diversity of the cadA gene in the microbial community in the Seine estuary

The diversity of the cadA gene sequences in the culturable bacteria was studied by RFLP analysis using the restriction enzyme *RsaI*. Five RFLP classes were identified (Fig. 2). Class I isolates yielded two *RsaI* fragments (800 bp and 258 bp), as did the *S. aureus* cadA gene present in Tn554. Only *Staphylococcus* species were found in this group. Class II isolates yielded three *RsaI* fragments (534 bp, 269 bp and 265 bp), like *S. aureus* pl258 cadA. This was the most abundant class, both in culturable bacteria (nine staphylococci, one *Bacillus* [MSA 236] and one *Micrococcus* [MSA 231] strain) and in cadA sequences directly amplified and cloned from total DNA of estuarine samples. Indeed, DNA sequences of 10 independent cadA genes recovered from the environment were 98 to 99% identical to that of the *S. aureus* (pl258) cadA gene (data not shown). Class III isolates generated four *RsaI* fragments (453 bp, 265 bp, 250 bp, 80 bp), as the pattern observed for the cadA from *B. firmus* [25]. This class contained *Bacillus* strain MSA 233. The *Bacillus* MSA 235 and *Halobacillus trueperi* MSA 234 strains belonged to two other RFLP classes (classes IV and V, respectively), which were dissimilar to all previously sequenced cadA genes.

A dendrogram based on a distance matrix was generated by comparing the CadA protein sequences deduced from the 1058-bp cadA region of our isolates with previously determined CadA sequences (Fig. 3). Eleven CadA sequences, representing the five *RsaI* restriction profiles and also one representative cadA sequence (clone D2) amplified from total DNA were chosen. The resulting phylogenetic tree exhibited three distinct clusters. Cluster A cor-

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**Fig. 1.** Map showing the sampling sites in the Seine estuary. A: The major treated wastewater effluent (two sampling series). B: The water column located at the mouth of the river (three sampling series). C: The sediments of the north intertidal mudflats (one sampling series). D: Mussels at the Marel station in the Seine bay filtering particles from both upstream and downstream of the estuary (one sampling series).
Table 1  
Bacteria isolated from the Seine estuary and carrying the cadA gene

<table>
<thead>
<tr>
<th>Strains</th>
<th>Phenotypic (P) or genotypic (G) identification</th>
<th>Sampling sites</th>
<th>cadA gene localization</th>
<th>RFLP class of cadA</th>
<th>IS257* between cadCA operon and an upstream IS257 (D1)*</th>
<th>Estimated distance (bp) between cadCA operon and a downstream IS257 (D2)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSA 100</td>
<td>S. aureus (7 different phenotypes)</td>
<td>Mussels</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>300</td>
</tr>
<tr>
<td>MSA 201</td>
<td>S. aureus 98.7% (P)</td>
<td>Estuary water</td>
<td>plasmid</td>
<td>I</td>
<td>+</td>
<td>300</td>
</tr>
<tr>
<td>MSA 202</td>
<td>S. aureus 98.7% (P)</td>
<td>Estuary water</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>300</td>
</tr>
<tr>
<td>MSA 203</td>
<td>S. aureus 96.2% (P)</td>
<td>Estuary water</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>300</td>
</tr>
<tr>
<td>MSA 204</td>
<td>S. capitis 90.9% (P)</td>
<td>Estuary water</td>
<td>plasmid</td>
<td>II</td>
<td>+ 1280</td>
<td>1000</td>
</tr>
<tr>
<td>MSA 205</td>
<td>S. cohnii 100% (P)</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>+</td>
<td>+ 280</td>
<td>–</td>
</tr>
<tr>
<td>MSA 206</td>
<td>S. cohnii 100% (P)</td>
<td>Estuary water</td>
<td>plasmid</td>
<td>II</td>
<td>+ 780</td>
<td>300</td>
</tr>
<tr>
<td>MSA 207</td>
<td>S. epidermidis 80.5%</td>
<td>Estuary water</td>
<td>plasmid</td>
<td>II</td>
<td>+ 780</td>
<td>300</td>
</tr>
<tr>
<td>MSA 208</td>
<td>S. epidermidis 97.9%</td>
<td>Estuary water</td>
<td>Nd</td>
<td>+</td>
<td>+ 780</td>
<td>800</td>
</tr>
<tr>
<td>MSA 209</td>
<td>S. epidermidis 99.2%</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MSA 210</td>
<td>S. epidermidis 99.3%</td>
<td>Estuary water</td>
<td>Nd</td>
<td>+</td>
<td>+ 1280</td>
<td>–</td>
</tr>
<tr>
<td>MSA 211</td>
<td>S. equorum 100% (P)</td>
<td>Estuary water</td>
<td>plasmid</td>
<td>II</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 212</td>
<td>S. equorum 100% (P)</td>
<td>Wastewater treated effluent</td>
<td>plasmid</td>
<td>+</td>
<td>+</td>
<td>2800</td>
</tr>
<tr>
<td>MSA 213</td>
<td>S. haemolyticus 99% (P)</td>
<td>Mudsflats</td>
<td>plasmid</td>
<td>II</td>
<td>+ 780</td>
<td>300</td>
</tr>
<tr>
<td>MSA 214</td>
<td>S. haemolyticus 99% (P)</td>
<td>Estuary water</td>
<td>plasmid</td>
<td>II</td>
<td>+ 780</td>
<td>800 and 2800</td>
</tr>
<tr>
<td>MSA 215</td>
<td>S. hominis 84.8% (P)</td>
<td>Estuary water</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MSA 216</td>
<td>S. hominis 95.3% (P)</td>
<td>Estuary water</td>
<td>plasmid</td>
<td>I</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MSA 217</td>
<td>S. hominis 95.3% (P)</td>
<td>Estuary water</td>
<td>Nd</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 218</td>
<td>S. hominis 84.8% (P)</td>
<td>Estuary water</td>
<td>Nd</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 219</td>
<td>S. lentus 99.8% (P)</td>
<td>Wastewater treated effluent</td>
<td>plasmid</td>
<td>II</td>
<td>+ 780</td>
<td>300</td>
</tr>
<tr>
<td>MSA 220</td>
<td>S. lentus 99.9% (P)</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MSA 221</td>
<td>S. saprophyticus 100% (P)</td>
<td>Wastewater treated effluent</td>
<td>plasmid</td>
<td>II</td>
<td>+ 780</td>
<td>300</td>
</tr>
<tr>
<td>MSA 222</td>
<td>S. sciuri 86.4% (P)</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 223</td>
<td>S. sciuri 92.6% (P)</td>
<td>Estuary water</td>
<td>Nd</td>
<td>+</td>
<td>+ 780</td>
<td>300</td>
</tr>
<tr>
<td>MSA 224</td>
<td>S. sciuri 99.3% (P)</td>
<td>Wastewater treated effluent</td>
<td>plasmid</td>
<td>II</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MSA 225</td>
<td>S. xylosoxy 99.9% (P)</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MSA 226</td>
<td>S. xylosoxy 99.9% (P)</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MSA 227</td>
<td>S. xylosoxy 99.9% (P)</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MSA 228</td>
<td>S. warneri 72.9% (P)</td>
<td>Estuary water</td>
<td>Nd</td>
<td>+</td>
<td>+ 3280</td>
<td>–</td>
</tr>
<tr>
<td>MSA 229</td>
<td>S. warneri 72.9% (P)</td>
<td>Estuary water</td>
<td>Nd</td>
<td>+</td>
<td>+ 3280</td>
<td>–</td>
</tr>
<tr>
<td>MSA 230</td>
<td>S. warneri 72.9% (P)</td>
<td>Estuary water</td>
<td>plasmid</td>
<td>I</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 231</td>
<td>Micrococcus spp. 99.9% (P)</td>
<td>Wastewater treated effluent</td>
<td>plasmid</td>
<td>II</td>
<td>+ 3280</td>
<td>–</td>
</tr>
<tr>
<td>MSA 232</td>
<td>Micrococcus spp. 99.9% (P)</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 233</td>
<td>Bacillus sp. MB-1 100% [AF326359 (G)]</td>
<td>Wastewater treated effluent</td>
<td>Nd</td>
<td>III</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 234</td>
<td>Halobacillus trueperi 96% [AJ310149 (G)]</td>
<td>Estuary water</td>
<td>chromosome</td>
<td>V</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 235</td>
<td>Bacillus sp. KL-152 100% [AY030033 (G)]</td>
<td>Estuary water</td>
<td>chromosome</td>
<td>IV</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSA 236</td>
<td>Bacillus sp. MB-1 97% [AF326359 (G)]</td>
<td>Mudsflats</td>
<td>Nd</td>
<td>II</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Bacteria resistant to 500 μM cadmium were isolated from water samples, sediment samples and mussels collected from the Seine estuary during seven independent sampling series. Bacteria harboring the cadA gene were detected by hybridization using the cadA probe gene.

*MSA, laboratory strains.

*The identification/relationship percentages correspond to either the API (Biomerieux) phenotypic (P) score obtained for a given strain, or to the percentage of identity between its 16S rDNA and that of reference strains (genotypic identification, G). In a few cases, the accession number of the reference strains is indicated in brackets.

*The localization of the cadA genes was determined by Southern blotting of plasmid DNA or after PCR amplification of the cadA gene from plasmid extracts of bacteria. Nd: not done.

*The RFLP class of the cadA genes (see Fig. 2) of each isolated species is indicated (I to V).

*The IS257 insertion sequences were detected by PCR with the OG1 primer. ^: no detectable PCR signal.

*The distances between the IS257 insertion sequences and the cadCA operon on the bacterial genome were determined as described in the legend of Fig. 5.
responded to RFLP classes I and II. It included the CadA sequence of *S. aureus* strain MSA 201, which is 91% identical to that of Tn554 from *S. aureus*, and the CadA sequence in several *Staphylococcus* and *Bacillus* (MSA 236) strains, and also the D2 clone, all closely related to pI258 CadA from *S. aureus*. Cluster B grouped RFLP classes III and IV found in the *Bacillus* strains MSA 233 and MSA 235, which were 80% identical to the *B. firmus* CadA. Cluster C included the CadA RFLP class V from *H. trueperi* (MSA 234), which is related to the CadA sequences of *L. lactis* and *L. monocytogenes*. The dendrogram also revealed that the predominant cadA sequences of this estuarine environment fall into a cluster of extremely well-conserved sequences (mostly RFLP class II). Interestingly, not only were these CadA present in four distinct staphylococci isolates, but one was found in a *Bacillus* sp. isolate. This strongly suggested the existence of horizontal gene transfer among these bacteria.

3.3. Genomic location of the cadA gene in bacterial species isolated from the Seine estuary

PCR and hybridization experiments were performed to determine whether the cadA genes were located on plasmids or on the chromosome in 17 selected strains from the Seine estuary. Plasmid DNA from nine different *Staphylococcus* and one *Micrococcus* isolate was analyzed by cadA-based PCR (data not shown). The cadA gene was plasmid-borne in the nine *Staphylococcus* strains and in the *Micrococcus* strain. Plasmid DNA from five *Staphylococcus*, one *Bacillus* and one *Halobacillus* strains was then subjected to hybridization. The cadA probe hybridized with one or more plasmid molecules of over 21 kb in bacteria belonging to the *Staphylococcus* genus, but with none of the plasmid forms extracted from *Bacillus (MSA 235)* or *Halobacillus (MSA 234)* (Fig. 4). Furthermore, no PCR signal was obtained for cadA using the plasmid DNA extracted from the *Bacillus* or *Halobacillus* strains, whereas the appropriate PCR products were observed with total DNA (data not shown). Thus, all of the *Staphylococcus* and *Micrococcus* species studied carried the cadA gene on plasmids, whereas the cadA gene is probably chromosomal in members of the *Bacillus (MSA 235)* and *Halobacillus (MSA 234)* genera (Table 1).

In order to assess the role of cadA in the cadmium resistance displayed by these bacteria, a curing experiment...
was performed with the *S. haemolyticus* (MSA 213) and the *S. saprophyticus* (MSA 221) strains harboring a single plasmid-borne copy of *cadA*. After incubation at 44°C for 48–72 h on LB medium, the resulting strains were unable to grow on R2A medium supplemented with 100 μM CdCl₂ (data not shown). Moreover, no plasmid could be observed in DNA prepared from these isolates, and PCR failed to amplify the *cadA* gene. These data are consistent with *cadA* being the major plasmid-borne cadmium resistance determinant in these bacteria.

### 3.4. Detection of IS257 insertion sequences near the *cadA* gene of *staphylococci*

The genomes of staphylococcal strains containing the *cadA* gene, isolated from the Seine estuary, were analyzed to evaluate the contribution of IS257 elements in the spread of CdR genes. The first experiment was designed to amplify IS257 elements by PCR using primer OG1. This primer corresponds to the inverted repeat (IR) of IS257 (Fig. 5A). Most of the *Staphylococcus* (25/31) and *Micrococcus* (1/2) strains analyzed (Table 1) contained at...
Fig. 5. Physical distances between the cadA gene and IS257 copies. A: Schematic representation of the S. aureus cadCA operon (located on pI258) flanked by two hypothetical IS257 copies. The ORFs of the cadCA operon are represented by rectangles. The ORFs of the cadCA operon are represented by rectangles. The OG1 primer (C) matches an 18-bp region of both invert repeat sequences (Q), and gives rise to an 800-bp PCR product. The invOG1 primer (C) is the inverse sequence of OG1. The cad1 and cad2 primers (C) amplified a 1058-bp internal fragment of the cadA gene sequence. To detect IS257, PCR amplification was first carried out with the OG1 primer. Two PCR amplifications were then carried out to determine whether the IS257 sequences were close to the cadCA operon. The first amplification was for the sequence located between the cad1 and invOG1 primers (d1), and the second amplified the segment between the cad2 and invOG1 primers (d2). The cadCA operon coordinates are those described for pI258. B: Molecular detection of IS257 sequences near the cadA genes in Staphylococcus. Electrophoresis gel showing the PCR products obtained with the cad1-invOG1 (a) and cad2-invOG1 (b) primer pairs using plasmid DNA (lanes 3 to 6) from cadA-positive strains. Lanes 1, Smartladder (Eurogentec); 2, negative PCR control; 3, S. equorum (MSA 211); 4, S. haemolyticus (MSA 214); 5, S. saprophyticus (MSA 221); 6, S. aureus (MSA 213).
least one IS257 sequence. In contrast, no IS257 elements were detected in the four Bacillus and Halobacillus strains.

Two PCR assays were then set up to determine whether IS257 elements were located near the cadCA operon of CdR Staphylococcus strains. The first experiment was designed to amplify the region between the cadmium-binding domain of CadA (primer cad1) and any IS257 (using primer invOG1) downstream of the operon (region d1 in Fig. 5). Conversely, a second assay was used to amplify the region located between the cadmium-channel domain of CadA (primer cad2) and any upstream IS257 (region d2 in Fig. 5). According to the DNA sequence of the pI258 cadA gene, the predicted length between the cad1 primer and the 3’ end of the cadCA operon is 1723 bp, and that between the cad2 primer and the 3’ end of the cadCA operon is 2192 bp. Therefore, the distances between any IS257 located upstream (D2) or downstream (D1) of the cadCA operon can be estimated as follows: D1 = |d1 − 1723 bp|, and D2 = |d2 − 2192 bp| (Fig. 5A). Fig. 5B shows the results obtained for four Staphylococcus cadA-strains.

For two S. haemolyticus strains (MSA 213 and MSA 214; Fig. 5B, lanes 3 and 6 [a,b]), and S. saprophyticus (MSA 221; Fig. 5B, lane 4 [a,b]), PCR products of 2.5 to 3 kb were obtained, suggesting the presence of IS257 elements flanking the cadA gene in these strains. The additional 1-kb fragment (lanes 3 and 4) most likely corresponded to the intervening sequence between two IS257 copies, since control PCR experiments performed with invOG1 primers alone gave rise to the same products (data not shown). In contrast, the S. equorum (MSA 211) strain did not harbor IS257 sequences near the cadA (Fig. 5B).

In one Micrococcus and 16 Staphylococcus strains, an IS257 sequence was located either upstream (4), downstream (4), or both (9) of the cadA gene (Table 1). The intervening segments between the cadCA operon and IS257 varied in length between 280 and 2800 bp. Interestingly, the lengths of some of these intervening sequences were found to be identical in different isolates, suggesting conservation of the regions surrounding the cadA gene (e.g. the 780 and 300-bp fragments observed in S. cohnii, S. epidermidis, S. haemolyticus, S. lentus and S. saprophyticus).

4. Discussion

The cadA gene, which confers resistance to cadmium, has been reported in four Gram-positive bacteria (S. aureus, B. firmus, L. lactis and L. monocytogenes) in both plasmid and chromosomal locations [10,25,50]. We have now identified the cadA gene in 11 different species of Staphylococcus, in addition to S. aureus, and showed, for the first time, that members of the Micrococcus and Halobacillus genera also harbor this gene.

Treated wastewater effluent and water from the mouth of the Seine contained numerous bacterial species harboring the cadA gene, notwithstanding the fact that cadA-bacteria have not previously been described in aquatic environments. Conversely, only a small number of different species carrying the gene were found in sediments. Most of the cadA genes in the bacteria isolated from the Seine estuary belonged to RFLP class II, as is the case for most of the cadA genes in the total DNA extracted from the water and from sediments. All these class II sequences displayed high identity (98–99%) with the pI258 cadA gene from S. aureus. This is consistent with a recent spread of this gene not only among staphylolococci, but also toward the Bacillus genus [51].

We found that the cadA gene is mostly plasmid-borne in members of the Staphylococcus and Micrococcus genera, whereas in members of the Halobacillus and Bacillus genera the cadA gene is probably chromosomal, as previously described for B. firmus [25]. It is important to note that in several cases, the precise determination of the bacterial host could not be obtained, and only distant relationship with existing species is given (Table 1). It is also likely that other bacteria from this natural environment, difficult to isolate or to maintain as was the case for our two ‘unculturable’ Bacillus strains, also carried the cadA gene [52].

Plasmids are one of the major driving forces involved in genetic transfer. They play a fundamental role in the spread of genes by conjugation [9,53]. This phenomenon occurs at frequencies of between 10^-5 and 10^-7 [53]. Nevertheless, the staphylococcal plasmids that carry heavy metal resistance genes are thought to be non-conjugative [4,9]. In this study, mixed-culture transfer experiments on solid medium did not give any evidence of conjugal phenomena for the staphylococcal plasmids containing the cadA gene (frequency < 10^-9; data not shown). However, the transfer behavior in natural environments remains unknown.

IS257 insertion sequences are able to transfer resistance genes within the Staphylococcus genus [4,6,7,15,54]. Insertion sequences are also involved in transposition events and homologous recombination [55]. Sixteen of our 31 Staphylococcus strains and a Micrococcus strain isolated from the Seine estuary carried at least one IS257 sequence close to the cadA gene. In nine cases, the cadA gene is probably flanked by IS257 sequences. The distances between the IS257 sequence and the cadCA operon were compatible with the cadA gene being carried by IS257-based composite transposons. This is reminiscent of the antibiotic resistance transposable elements previously described in staphyloccoci. The 4.7-kb composite transposon Tn4003 is flanked by IS257 and is involved in trimethoprim resistance (drfA), and the 7.5-kb element Tn4291, also flanked by IS257, is involved in methicillin resistance (mecA) [4]. Similarly, IS257 sequences have previously been found in the vicinity of heavy metal resistance genes. Four IS257 have been detected near the chromosomal mercury resistance genes (merA and merB) and near the mer gene of pI258 in methicillin-resistant S. aureus [4,54].
A novel cadmium resistance gene, cadD, closely related to the cadB gene and implicated in the biosequestration of toxic Cd\textsuperscript{2+} ions, was described in S. aureus [15]. Sequence analysis showed that the cadmium resistance operon cadDX is also located on a 3972-bp DNA fragment flanked by IS257 sequences [15]. Finally, Alonso et al. [26] stated that the flanking IS257 sequences are the only explanation for the presence of a cadA gene, which is 98% identical to the staphylococcal cadA gene from pl258, in the Gram-negative S. maltophilia.

In conclusion, most of the cadA genes isolated from the microbial community of the Seine estuary were found to be almost identical to each other. Surprisingly, their host bacteria were members of different species or even genera, and most have never previously been reported to be carriers of the cadA gene. For the Staphylococcus and Micrococcus species, the presence of plasmids harboring the cadA gene flanked by IS257 insertion sequences could be responsible of the spread of this cadmium resistance gene in this aquatic environment.

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