**INTRODUCTION**

*Streptococcus pneumoniae* is a human pathogen and the major cause of acute bacterial pneumonia and otitis media. Approximately one million deaths worldwide are attributed to this bacterium each year. Its main virulence factor is the polysaccharide capsule, which enables the bacterium to evade host defences. As many as 90 different capsular types have been identified, suggesting that this species displays a high level of genetic plasticity. This phenomenon may be stimulated by the natural transformation capacity of *S. pneumoniae* (for a review, see Claverys et al., 2000).

The extent of genetic plasticity displayed by *S. pneumoniae* raises the question of whether cellular processes other than natural transformation are involved. One process known to generate plasticity is double-strand DNA break (DSB) repair by homologous recombination. This process sometimes leads to chromosomal rearrangements and is thus a potential cause of genomic plasticity (Gruss & Michel, 2001). In Gram-positive bacteria, the exonuclease/helicase RexAB is a major component of the homologous recombination process and is essential for DSB repair (Chedin & Kowalczykowski, 2002; Quiberoni et al., 2001b). It has been studied in *Bacillus subtilis* (where it is called AddAB; Doly et al., 1974; Kooistra & Venema, 1991) and in *Lactococcus lactis* (El Karoui et al., 1998), and is the functional homologue of the RecBCD enzyme of *Escherichia coli*. Unlike RecBCD, which has a single nuclease (and two helicases) to degrade both strands, the RexAB enzyme comprises two distinct nuclease activities that appear to each degrade one of the two strands (Quiberoni et al., 2001a). Nuclease activities have been mapped to motifs present at the C-terminal extremities of both RexA and RexB subunits (Quiberoni et al., 2001a), which are conserved in all RexAB homologues examined so far (Quiberoni et al., 2001b). RexAB exonuclease activity degrades DNA from a double-strand end, but is attenuated when it encounters a short DNA sequence known as Chi (Chedin et al., 1998; El Karoui et al., 1998). After a Chi encounter, the helicase activity remains (Chedin et al., 2000), leading to formation of a 3'-OH single-strand extremity (the preferential substrate for RecA-mediated homologous pairing reaction) and subsequent repair of the molecule by homologous recombination. The sequence comprising Chi has been shown to vary according to the species (*E. coli*, *L. lactis*, *Haemophilus influenzae* and *B. subtilis*; for review see El Karoui et al., 1999). It is interesting to note that despite the importance of exonuclease/helicase-Chi couples in genome integrity via DSB repair, the structure of their components is only poorly conserved.

To characterize the role of RexAB in *S. pneumoniae*, we constructed rexAB insertion mutants and examined the phenotypes of these mutants with respect to growth, DNA repair capacity and recombination in gene conversion. AddAB has been proposed in *B. subtilis* to have an additional role in recombination during natural transformation (Kooistra et al., 1988), but this observation seems to be strain-dependent (Fernandez et al., 2000). To clarify this issue we also tested the natural transformation ability of our rexAB mutants.
METHODS

Bacterial strains, growth conditions and competence. The bacterial strains used in this study are all derivatives of R800 (Tiraby et al., 1975). Construction of a lytA derivative of R800: an erythromycin resistance cassette (from plasmid pR409; Martin et al., 2000) was introduced into the lytA gene carried by plasmid pGL32 (Lopez et al., 1986) via partial EcoRI digestion. This plasmid was transformed into R800 to interrupt the lytA chromosomal copy by double crossing over. The phenotype of the resulting strain, DH39, was verified by testing its resistance to deoxycholate-induced lysis (Garcia et al., 1985). The lytA::erm construct was then introduced by transformation into rexAB strains DHPI4 and D15 (see next paragraph) giving rise to strains DHP45 and DHP43, respectively. The rexA derivative of R800 that we used is called R209 (Martin et al., 1995).

In vitro mariner mutagenesis. Mutagenesis was carried out essentially as described previously (Martin et al., 2000). A 7·3 kb DNA fragment encoding rexAB was PCR-amplified using primers ODH9 and ODH17 (ODH9, 5′-ATTCCGAGTCTTTGACAGAA-3′ at the beginning of rexB; ODH17, 5′-ATTCCTTTGTAGCCTTTGTCCCC-3′ at the end of rexA), and strain R800 chromosomal DNA as template. Plasmid pR412 (Martin et al., 2000) was used as a source of the SpcR mariner minitransposon to tagenate the rexAB fragment in vitro, which was then transformed into R800. Ten SpcR transformants were checked by PCR and shown to carry a mariner insertion. Two of them (strains DHPI4 and DHPI5) were further analysed; the precise insertion position was determined by PCR and DNA sequencing using primers ODH9, ODH17, MP127 and MP128 (MP127, 5′-CGGAGGACTTTATACGCAACC-3′; MP128, 5′-TACGT-AGCGAGCCCATCTATG-3′; Martin et al., 2000).

UV irradiation. Cells were grown in liquid culture to exponential phase to an OD550 of approximately 0·1. Dilutions were then plated and cells were UV-irradiated at 5, 10 and 25 J m−2 using a UV stratalinker (Stratagene; the wavelength was 254 nm and the energy was calculated according to the manufacturer’s protocol – see www.stratagene.com/manuals/7003406.pdf). Survival was assessed after 24 h incubation at 37 °C.

High molecular weight (HMW) plasmid detection. HMW multimer accumulation was detected as described by Sourie et al. (1998). Total DNA was prepared using cells grown to an OD550 of approximately 0·2 with the Qiagen Genomic-tip 20/G kit (according to the manufacturer’s protocol, except that 10 mg lysozyme ml−1 and 50 U mutanolysin ml−1 were added to facilitate cell lysis). HMW multimers were detected by Southern blot hybridization using a plasmid-specific probe. The Southern blot was scanned and the ratio of HMW versus total plasmid DNA content was quantified using ImageQuant v5 software.

RESULTS AND DISCUSSION

Construction of rexAB mutants by in vitro mariner transposition

Analysis of the preliminary sequence data from the complete genome sequencing project of strain G54 (Dopazo et al., 2001) indicated the presence of S. pneumoniae RexA and RexB proteins 41 and 29% identical, respectively, to their L. lactis homologues. We made use of these data to construct SpcR rexAB insertion mutants by in vitro mariner mutagenesis, as described in Methods. The DHPI4 insertion is in rexB (TA target, 2614 nt from the ATG start codon at aa 871; transcription of the spc cassette is in the opposite sense compared to rexB, so its promoter cannot induce rexA mRNA production); the DHPI5 insertion is in rexA (TA target, 2768 nt from the ATG start codon at aa 923; the spc cassette is co-transcribed with respect to rexA; Fig. 1).

The insertion in rexA is upstream of the nuclease motif that starts at aa 1148 (Fig. 1). In L. lactis, inactivation of this motif leads to a total loss of RexAB activity (Quiberoni et al., 2001a). The rexAB genes appear to form an operon and a putative extended −10 promoter was identified upstream of rexB (Fig. 1). It is thus likely that, as in L. lactis, the rexB insertion is polar on rexA (El Karoui et al., 1998). It was previously shown that RexA is absolutely required for all RexAB activities in B. subtilis and in L. lactis (Hajjema et al., 1996; Quiberoni et al., 2001a). We therefore hypothesized that both mutants display a rexAB-null phenotype. Analyses of the S. pneumoniae rexAB mutants confirming this hypothesis are presented below.

Inactivation of rexAB affects growth and DNA repair

RexAB is a major component of the DSB repair machinery. DSB occurs during the course of normal cellular processes like chromosome replication (Gruss & Michel, 2001) or because of exposure to DNA-damaging agents (Thoms & Wackernagel, 1998). As the accumulation of non-repaired DSB during growth usually leads to cell death, rexAB mutants are expected to display reduced growth capacity and reduced survival after exposure to DNA-damaging agents.

Both rexA (strain DHPI5) and rexB (strain DHPI4) were severely impaired for growth, as monitored by OD550.

![Fig. 1. Location of insertions in the rexAB operon. The rexB and rexA genes are indicated by large grey arrows and the putative promoter is shown. Locations of the transposon insertions are shown by triangles, above which the corresponding strain number is indicated. The flags indicate the direction of SpcR cassette transcription in the mariner transposon. The pale grey boxes indicate the positions of nuclease motifs. Primers used for analyses are indicated by small arrows with their identification numbers.](image-url)
measurements (Fig. 2, filled symbols). This may suggest that only a fraction of the cell population is viable. Indeed, for a given OD550, the numbers of colony-forming centres (CFC) of both rexAB mutants were lower than those obtained with the wild-type (wt) strain (Fig. 2, open symbols). rexA and rexB mutant viability (CFC per OD550 unit) was around 20% of that of the wt strain (calculated from data in Fig. 2). These results are in keeping with viabilities reported for E. coli recBCD or B. subtilis addAB mutant strains (Capaldo et al., 1974; Kooistra et al., 1988). The observed lower ratio of CFC to OD550 in the rexAB strains could possibly reflect the presence of elongated cells due to the mutation. However, examination of wt and mutant strains by microscopy revealed no differences in cell shape or size: the ratio of diplococci to tetracocci and longer chains was identical (data not shown). Cell length measurements further confirmed that the rexAB mutation had no effect on S. pneumoniae cell size; the mean cell length was 1.01 ± 0.2 μm for the wt strain and 0.98 ± 0.18 μm for both rexAB mutants. Note that both wt and rexAB S. pneumoniae cells died quickly after reaching stationary phase (as deduced from the rapid decrease in CFC) while OD550 remained constant.

UV irradiation induces DNA damage that is repaired by excision repair, which creates single-strand interruptions in the DNA molecule. If a replication fork runs into such single-strand breaks, DSBs are formed (Kuzminov, 1995, 2001). We examined the UV sensitivities of wt and rexAB strains DHP14 and DHP15, as well as of a S. pneumoniae recA strain, R209 (see Methods). Our results show that UV sensitivity of the rexAB mutant strains is intermediate between that of the recA and the wt strains (Fig. 3). These results are in keeping with what we previously observed in L. lactis (El Karoui et al., 1998). UV sensitivity thus appears to be a general feature of rexAB mutants in Gram-positive low-GC bacteria (Kooistra et al., 1988). The above results indicate that inactivation of rexAB results in a reduced ability to repair DNA damage in S. pneumoniae.

**rexAB mutants are not affected in recombination during natural competence or in plasmid establishment**

Natural transformation involves DNA uptake, followed by its integration via homologous recombination. In Gram-positive low-GC bacteria, DNA is internalized as a linear single-strand molecule (Dubnau, 1999) that can be directly aligned with its genomic homologue by a RecA-mediated reaction. One role of the RexAB exonuclease/helicase is to generate a single-strand recombinogenic substrate for RecA, starting from a double-strand substrate. As the internalized DNA is single-strand, RexAB is not expected to be involved in the transformation process. Nevertheless, expression of addAB (the rexAB homologue) in B. subtilis is reportedly induced during competence in a ComK-dependent manner (Hajijema et al., 1995) and is needed for chromosomal transformation (Kooistra et al., 1988). We examined the role of rexAB in chromosomal DNA transformation in S. pneumoniae. The transformation frequencies of rexAB versus wt strains differed by less than threefold (Table 1). We consider this difference as non-significant, as competence efficiency of a wt strain can vary from batch to batch by a factor of up to 10-fold (we observed a broad standard deviation, as seen in Table 1). These data suggest that RexAB is not involved in genetic recombination during transformation with chromosomal DNA in S. pneumoniae. Although addAB has been reported as necessary for chromosomal transformation in strain 8G5 of B. subtilis (Kooistra et al., 1988), it is not the case in another genetic
The proportion of StrR colonies was corrected for the number of viable cells. Cultures were inoculated with alleles of the assay was performed in the R960 strain, which carries two previously developed test system (Sung et al., 2001). We introduced the rexA and rexB mutations into the R960 background; the gene conversion frequency was determined as the proportion of StrR cells among the total number of viable cells in an exponentially grown culture. The conversion frequency was slightly reduced in rexAB mutants (~three- and fivefold for rexB and rexA, respectively; Table 2) compared to the ~40- to 200-fold reduction observed in a recA mutant (Sung et al., 2001). While the reduction was slight, we consider it significant, as the standard deviations observed between independent measurements were very small. These results may suggest that 50–80% of the conversion events are RexA-dependent. Nevertheless, other tests would be required to confirm the biological importance of RexAB in gene conversion events. We conclude that RexAB is not a major player in gene conversion in S. pneumoniae, and at least another RexA-independent pathway is likely to exist to initiate conversion. This is not surprising in light of the multiple pathways identified in other bacteria that generate single-strand substrates for RecA annealing. For example, gene conversion in E. coli is mediated by the RecBCD pathway (Zieg & Kusher, 1977), but can also be mediated by the RecF or RecE pathway (Kobayashi, 1992).

### Double-strand exonuclease activity is attenuated in rexAB mutants

We previously established that rolling-circle plasmids form HMW multimers in strains deficient for double-strand exonuclease activity (Biswas et al., 1995; Dabert et al., 1992). Degradation of HMW multimers requires the presence of intact nuclease motifs on the RexAB enzyme (Quiberoni et al., 2001a). Insertion sites of our rexA and rexB S. pneumoniae mutants should inactivate one or both nuclease motifs, respectively. This system was therefore used to follow exonuclease activity in the S. pneumoniae mutants. We encountered difficulties in testing for HMW multimers in rexAB strains DHP14 and DHP15 due to their very slow growth and quick lysis. To overcome problems of DNA degradation associated with lysis, the major pneumococcal

### Table 1. rexAB mutants are not affected in recombination during natural competence

<table>
<thead>
<tr>
<th>Strain (rexAB allele)</th>
<th>Transformation efficiency (%)*</th>
<th>Chromosomal DNA†</th>
<th>Plasmid DNA‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>R800 (wt)</td>
<td>2.6±2.6</td>
<td>0.07±0.03</td>
<td></td>
</tr>
<tr>
<td>DHP14 (rexB::spc)</td>
<td>1.45±0.4</td>
<td>0.09±0.017</td>
<td></td>
</tr>
<tr>
<td>DHP15 (rexA::spc)</td>
<td>1±0.8</td>
<td>0.03±0.023</td>
<td></td>
</tr>
</tbody>
</table>

*The yield of StrR transformants (chromosomal transformation) and chloramphenicol-resistant (plasmid transformation) transformants was corrected for cell viability.
†Each result corresponds to the mean ±SD of 4–8 experiments.
‡Each result corresponds to the mean ±SD of 3–4 experiments.

### RexAB inactivation has a slight effect on gene conversion

As a component of a homologous recombination pathway, RexAB might be involved in recombination events occurring during gene conversion. We tested whether rexAB mutants are affected in gene conversion using a previously developed test system (Sung et al., 2001). The assay was performed in the R960 strain, which carries two alleles of the rpsL gene, the dominant StrR rpsL wt allele (linked to a kanamycin resistance gene) and the Strr rpsLA1 allele. Thus R960 is normally Str+, but StrR clones arise at significant levels. It was previously shown that these clones are the result of a homologous recombination event between rpsLA1 and the rpsL wt allele (Sung et al., 2001). We previously established that rolling-circle plasmids form HMW multimers in strains deficient for double-strand exonuclease activity (Biswas et al., 1995; Dabert et al., 1992). Degradation of HMW multimers requires the presence of intact nuclease motifs on the RexAB enzyme (Quiberoni et al., 2001a). Insertion sites of our rexA and rexB S. pneumoniae mutants should inactivate one or both nuclease motifs, respectively. This system was therefore used to follow exonuclease activity in the S. pneumoniae mutants. We encountered difficulties in testing for HMW multimers in rexAB strains DHP14 and DHP15 due to their very slow growth and quick lysis. To overcome problems of DNA degradation associated with lysis, the major pneumococcal

### Table 2. rexAB mutants are slightly affected in gene conversion

<table>
<thead>
<tr>
<th>Strain (rexAB allele)</th>
<th>StrR frequency per 10⁶ cells*</th>
<th>-Fold reduction</th>
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<tbody>
<tr>
<td>R960 (wt)†</td>
<td>1.7±0.35</td>
<td>1</td>
</tr>
<tr>
<td>DHP58 (rexB::spc)†</td>
<td>0.62±0.19</td>
<td>2.7</td>
</tr>
<tr>
<td>DHP57 (rexA::spc)†</td>
<td>0.37±0.21</td>
<td>4.6</td>
</tr>
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</table>

*The proportion of StrR colonies was corrected for the number of viable cells. Cultures were inoculated with several StrR colonies, grown to an OD₅₅₀ of approximately 0-5, and plated with and without Str. Values are presented ±SD.
†Results are the means of 3 (wt strain), 4 (strain DHP58) or 5 (strain DHP57) experiments.
autolysin LytA (Lopez et al., 1986) was inactivated in R800, DHP14 (rexB) and DHP15 (rexA) strains giving rise, respectively, to DHP39, DHP45 and DHP43 (see Methods). We then introduced pJS3, a rolling-circle replicon, into strains DHP39, DHP43 and DHP45 giving rise to strains DHP60 (rexA lytA) and DHP62 (rexB lytA), respectively. The presence of plasmid-generated HMW multimers was detected by Southern blot hybridization of total DNA using a pJS3-specific probe. As shown in Fig. 4, HMW multimers accumulated in both DHP60 (rexA lytA) and DHP62 (rexB lytA), but not in DHP40 (lytA). Scanning of the Southern blot and quantification indicated that HMW multimers represented approximately 13 and 18 % of the total plasmid DNA content in DHP60 and DHP62, respectively, compared to about 1 % in DHP40. HMW multimer accumulation was slightly lower than that observed in rexAB mutants of other species (Chedin et al., 1998), which may indicate the existence of residual nucleolytic activity (possibly due to another nuclease present in S. pneumoniae). This result indicates that, as expected, double-strand exonuclease activity is attenuated in S. pneumoniae rexA and rexB mutants.

This characterization of rexAB mutants suggests that RexAB is involved in DSB repair in S. pneumoniae. RexAB is thus likely to play a key role in maintaining genome integrity in this species. Although RexAB does not seem to be involved in natural competence, it may nevertheless play a role in genome plasticity by catalysing homologous recombination events between repeated sequences (Oggoni & Claverys, 1999), particularly between the numerous IS elements present on the S. pneumoniae chromosome (Tettelin et al., 2001). We are presently identifying the S. pneumoniae Chi site to further characterize the factors that modulate RexAB activity. Interestingly, preliminary analysis shows that the Chi sequence of L. lactis is extremely frequent on the S. pneumoniae genome and is distributed with a strong strand bias, as is the case for the Chi sites of E. coli and L. lactis (El Karoui et al., 1999). It might therefore constitute a good candidate as the sequence potentially recognized as Chi in S. pneumoniae.

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