

# spr1630 Is Responsible for the Lethality of *clpX* Mutations in *Streptococcus pneumoniae*<sup>∇</sup>

Andrew Piotrowski,<sup>1</sup> Peter Burghout,<sup>2</sup> and Donald A. Morrison<sup>1\*</sup>

Laboratory for Molecular Biology, Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois 60607,<sup>1</sup> and Laboratory of Pediatric Infectious Diseases, Nijmegen Medical Centre, 6500 HB Nijmegen, The Netherlands<sup>2</sup>

Received 3 March 2009/Accepted 12 May 2009

**The Clp protease ATPase subunit and chaperone ClpX is dispensable in some bacteria, but it is thought to be essential in others, including streptococci and lactococci. We confirm that *clpX* is essential in the Rx strain of *Streptococcus pneumoniae* but show that the requirement for *clpX* can be relieved by point mutations, frame shifts, or deletion of the gene *spr1630*, which is found in many isolates of *S. pneumoniae*. Homologs occur frequently in *Staphylococcus aureus* as well as in a few strains of *Listeria monocytogenes*, *Lactobacillus johnsonii*, and *Lactobacillus rhamnosus*. In each case, the *spr1630* homolog is accompanied by a putative transcriptional regulator with an HTH DNA binding motif. In *S. pneumoniae*, the *spr1630-spr1629* gene pair, accompanied by a RUP element, occurs as an island inserted between the *trpA* and *cclA* genes in 15 of 22 sequenced genomes.**

The ATP-dependent proteases play important roles in bacterial physiology. In addition to monitoring protein quality and clearing damaged or misassembled proteins, they also participate in numerous regulatory pathways via specific proteolysis of regulatory proteins (13, 26, 32, 40). One family of these proteases is composed of a peptidase core, ClpP, complexed with one of a few ATPase specificity factors that provide for transport of protein substrates into the otherwise inaccessible core for proteolysis. While it thus might be expected that Clp protease mutations would usually be highly pleiotropic, the most prominent phenotype associated with loss of ClpP varies from one species to another. Thus, for example, *clpP* mutants of a number of low-GC gram-positive species have reduced thermo-tolerance (7, 11, 20, 28, 29, 35). A loss of virulence is the most prominent phenotypic effect of mutation of ClpP in *Staphylococcus aureus* (8), while in *Salmonella*, altered expression of flagellar operons is a major effect (43). In contrast, inactivation of *clpP* in *Caulobacter crescentus* is lethal (15). Some of these phenotypes have been traced to roles of ATP-dependent proteases in controlling the half-life of bacterial regulatory proteins, including the stationary phase sigma factor  $\sigma^S$  and the heat shock sigma factor  $\sigma^{32}$  in *Escherichia coli* (41, 49) and the sporulation sigma factor  $\sigma^H$  (22), the competence transcription factor ComK (44), the RsiW antisigma factor (48), the regulator Spx (30), and the Clp protease regulator CtsR in *Bacillus subtilis* (18).

Among the Clp ATPase subunits, ClpX is widely conserved among bacteria, but its role in bacterial physiology varies from one species to another. In some, it carries out essential functions, while in others its roles are more subtle. In *Caulobacter*, critical cell cycle regulators depend on proteolysis by ClpXP for correct timing of cell division (15). In *Synechococcus*, *Streptococcus*, and *Lactococcus*, ClpX also appears to be essential (5, 9, 37), but it is dispensable in other bacteria. In *Streptomy-*

*ces*, *clpX* mutants are viable but have a developmental defect revealed only at low pH (45). While *clpX* mutants of *B. subtilis* are heat sensitive (12), *clpX* mutants of *Staphylococcus aureus* are more tolerant of elevated temperatures (8, 10). The ClpX protein can also act in nonproteolytic roles, as when it retards assembly of the FtsZ ring (47), remodels the Mu phage replication complex (16, 21, 27, 46), or acts independently of ClpP in positive regulation of transcription of the protein A gene in *S. aureus* (8, 10).

The creation of ClpX mutants of *Streptococcus pneumoniae* (pneumococcus) was reported independently by Robertson et al. (35), using strain R6, and by Luo (23), using the related strain, Rx. However, Robertson et al. (36) subsequently reported that all apparent *clpX* mutants in their hands resulted from rare rearrangements in strain R6 that result in acquisition of a disrupted *clpX* gene but also in retention of an intact copy of the gene, creating a merozygote. Specifically, an R6 derivative was created that carries both a disrupted *clpX* gene and an ectopic copy of the intact gene expressed under the control of the fucose promoter. As the viability of this strain depends on the provision of fucose to ensure expression of the ectopic *clpX*, it is clear that *clpX* is an essential gene in strain R6, presumably reflecting some nonproteolytic role, as *clpP* is dispensable in this strain. We were curious as to whether CP1361, the *clpX* mutant obtained by replacing most of *clpX* with an *erm* marker in the Rx background (23), might have a similar complex explanation or might be truly deficient in *clpX*. Here we resolve this discrepancy by showing that the *clpX* deletion mutant CP1361 carries an unlinked suppressor mutation that permits normal growth in the absence of *clpX* and by mapping the suppressor mutation to a conserved genetic island found in most pneumococcal isolates.

## MATERIALS AND METHODS

**Strains used in this study.** Tables 1 and 2 list the strains and primers used in this study. CAT broth contained (per liter) 5 g of tryptone (Difco Laboratories), 10 g of enzymatic casein hydrolysate (ICN Nutritional Biochemicals), 1 g of yeast extract (Difco), and 5 g of NaCl. It was sterilized for 40 min at 121°C and then brought to 0.2% glucose and 1/60 (0.0167) M K<sub>2</sub>HPO<sub>4</sub> before use. Antibiotics were used at the following concentrations: 2.5 µg/ml for novobiocin (Nov), 0.25

\* Corresponding author. Mailing address: LMB, Room 4150, 900 South Ashland Ave., Chicago, IL 60607. Phone: (312) 996-6839. Fax: (312) 413-2691. E-mail: DAMorris@uic.edu.

<sup>∇</sup> Published ahead of print on 22 May 2009.

TABLE 1. Strains used in this study

Strain	Description	Reference, source, or construction <sup>a</sup>
CP1250	Rx derivative, low β-galactosidase activity; <i>hex malM511 str-1 bgl-1</i> ; Hex <sup>-</sup> Mal <sup>-</sup> Sm <sup>+</sup> Bga <sup>-</sup>	33
CP1361	CP1250, but Δ <i>clpX::erm slx-1</i> ; Em <sup>r</sup>	23
CP1500	Rx derivative; <i>hex novo-r brya-r str-r1 ery-r2 ery-r6</i> ; Nov <sup>r</sup> Em <sup>r</sup> Str <sup>r</sup>	4
CP1973	CP1361, but <i>clpX</i> <sup>+</sup> ; Em <sup>s</sup>	This study
CP2008	CP1250, but Δ <i>spr1630::kan</i> ; Kan <sup>r</sup>	This study
CP2086	R6, but <i>novo-r</i> ; Nov <sup>r</sup>	R6 × CP1500
CP2087	R6, but Δ <i>spr1630::kan</i> ; Kan <sup>r</sup>	R6 × CP2008
CP2088	R6, but Δ <i>spr1630 ΔclpX::erm</i> ; Kan <sup>r</sup> Em <sup>r</sup>	CP2087 × CP1361
CP2089	TIGR4, but Δ <i>spr1630::kan</i> ; Kan <sup>r</sup>	TIGR4 × CP2008
R6	Rough derivative of D39	14
TIGR4	Serotype 4 isolate	42

<sup>a</sup> Strain construction by transformation is indicated as recipient × DNA donor.

μg/ml for erythromycin (Em), 200 μg/ml for kanamycin (Kan), and 40 μg/ml for spectinomycin (Sp).

Creation of strain CP1361 (Δ*clpX::erm*) was as described previously (23, 34). Briefly, a fragment upstream of *clpX* was amplified from strain CP1500 using

primers PL72 and PL71, a downstream fragment was amplified from CP1500 using PL73 and PL74, and a third fragment containing the *erm* marker was amplified from amplicon aMSL1 (19) using PL46 and PL47. After digestion by XbaI and/or HindIII, the three fragments were purified, ligated, and used directly as donors for transforming strain CP1250, with selection for Em<sup>r</sup>. The mutation in a single clone, named CP1361, was confirmed by sequencing a fragment that was amplified using the outermost primers for creating the construct.

Strain CP2008 was created similarly by disruption of *spr1630*, except the upstream fragment was amplified using DAM862 and DAM865, the downstream fragment was amplified using DAM863 and DAM864, the *kan* insert was amplified from pR410 (38) using DAM301 and DAM302, and the fragments were digested by BamHI and/or ApaI. To create strain CP1973, a *clpX*<sup>+</sup> revertant of strain CP1361, we took advantage of the high transformation efficiency of large donor DNA amplicons to accomplish allele replacement without selection. A 7,736-bp *clpX*<sup>+</sup> donor fragment was amplified from CP1250 using AP30 and AP33. CP1361 was transformed using this fragment as a donor, as described below, but with an extended (3-h) period of incubation to allow for segregation of the new allele. Thirty resulting colonies were used individually to inoculate CAT and, in parallel, CAT with Em. Seven clones that grew in CAT, but not in the presence of Em, were analyzed by PCR, using primers AP22/25 and AP23/27 to determine which were *clpX*<sup>+</sup>. Of the seven clones, six appeared to be *clpX*<sup>+</sup> and one was retained as CP1973.

**PCR amplification.** PCR amplification was done by using commercially available PCR supermixes. PCR SuperMix high fidelity (Invitrogen) was used for fragments intended for sequencing or for transformation, while PCR SuperMix (Invitrogen) was used in other cases. Reaction mixtures (50 μl) contained 45 μl SuperMix, 0.5 μl of each primer (final concentration 500 nM), 1 μl of DNA

TABLE 2. Primers used in this study

Primer name	Gene	Primer sequence (5'–3') <sup>a</sup>
AP22	<i>clpX</i>	GATAGAATGACAGTATAGAATGGAAGGAAATC
AP23	<i>clpX</i>	ATTGTGAATGAATGCGTGGAGTTAGCTCAG
AP24	<i>clpX</i>	ATGCGTGGAGTTAGCTCAGGAAATCATTCCG
AP25	<i>clpX</i>	CTAGGATCGGTTTATCCGTTCCATCGACAG
AP26	<i>clpX</i>	TAGCAATCTCTTGAAGGGCTTCGTCGTCAAATTCC
AP27	<i>clpX</i>	ACGCGCCCCTGTCTTCCGTTCCGATTGCTTTATTAG
AP30	<i>spr1422</i>	CTTGCTGACTCAGGGTCACGAATG
AP33	<i>lytC</i>	AACGCAAACGAGCGCAGAAGTACAG
DAM301	<i>kan</i>	CGCGCAAGCTGGGGATCCG
DAM302	<i>kan</i>	acgtggcccTAGGTAATAAAAACAATTCATCCAGTAA
DAM850	<i>spr1620</i>	TGGAAGCTCTGATTCCGCAATTCCG
DAM851	<i>spr1627</i>	AGGCTTAGGTAGCCAGGCTTTAAGG
DAM852	<i>spr1623</i>	AGCTGGAGACAGCACTCCGTAGTTG
DAM853	<i>trpA</i>	GGTGACCACGAGAAAGGTTTGGATG
DAM854	<i>spr1630</i>	GTAACGTACCAGTTCCGTGCATCAGG
DAM855	<i>trpE</i>	GAAAGCAACGCTTCCTGCTGGAACC
DAM856	<i>trpD</i>	TCAAGCCAGCTTCATCCAACCCTTC
DAM857	<i>mgtC</i>	TCTGACAACCTGCAGCAGGCATTTGG
DAM858	<i>ccpA</i>	AGAACGCACACCTTTTCACGATCC
DAM859	<i>galE</i> <sup>b</sup>	TGACAGCGCTTGACAAAGGAAACGG
DAM860	<i>spr1645</i>	TTGCTAGAATTGCCCGTCTCTGTACC
DAM861	<i>spr1650</i>	ATCAACCGAGTGGGTACTGGATAGG
DAM862	<i>trpA</i>	CACCAAGGAGAGCCGATTCAGGAC
DAM863	<i>spr1629</i>	CCATGCGCTATAAGCATCAGCTTGG
DAM864	<i>spr1630</i>	gcaggatccCCTGATGCACGAACTGGTCAGTTAC
DAM865	<i>spr1630</i>	acaggcccAGGTCGCGTCCATGCATAACCCATTC
PL46	<i>erm</i>	GGTCTAGA GGATCCGGGTACCGGGCCAAAATTTGTTTGATTTGT
PL47	<i>erm</i>	GGGGTACCGAAGCTTGGGATCCGGAATTCAGTCGGCAGCGACTC ATAGAATTA
PL71	<i>clpX</i>	gctTAGACATGATTTCTTCCATTCTATACTG
PL72	<i>dpr</i>	CGTGTCTTGTTATCTACCGTTACT
PL73	<i>clpX</i>	cccaagctttcATGTTTGAGGTGCCGAGTCAGGA
PL74	<i>aldR</i>	TTACATCACGAGGAAGACGAGCTAC
SA130	<i>spc</i>	GATCTGTCAATGGTTCAGATAC
SA133	<i>spc</i>	GAAGAGCCATTATGGATTCCG

<sup>a</sup> Lowercase segments indicate 5' extensions; underlined segments indicate restriction sites.

<sup>b</sup> Primer binds upstream of *galE*.

(~100 ng), or 1  $\mu$ l cell culture at an optical density (OD) of 0.1, and 3  $\mu$ l sterile water. The PCR conditions were 95°C for 30 s; 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C (68°C for fragments >5 kbp in length) for 1 min/kbp target length; 72°C (or 68°C) for 10 min.

***slx marinerT7* library DNA.** To create an *slx marinerT7* transposon ( $\Omega$ *spc*) library, CP1973 was transformed with DNA from the *marinerT7* transposon library (2), as described below. The cells were plated on the surface of CAT agar containing Spc. After overnight incubation, ~150,000 Spc<sup>r</sup> colonies were washed off the surface of the agar and suspended in 300 ml complete CAT plus Spc. After growth for ~five generations, the 0.4-OD<sub>550</sub> culture was harvested by pelleting the cells (5,000  $\times$  g, 20 min, 4°C), washing them in 20 ml buffer A (0.1 M Tris-HCl [pH 7.6], 10 mM EDTA, 0.1 M NaCl), and resuspending them in 10 ml buffer A with Triton X-100 to 0.2%. After incubation at 37°C for 100 min, DNA was purified by chloroform extraction and ethanol precipitation, as described previously (24). DNA in the final 5-ml volume was estimated by quantitation of gel images after electrophoresis in 0.8% agarose gels.

**Inverse PCR.** Mini-DNA extracts were prepared by a similar procedure using 10 ml of each culture. To determine the position of *marinerT7* insertions, 1  $\mu$ g of genomic DNA was digested with 10 units of HpyCHIV (New England BioLabs) in 25  $\mu$ l NEBuffer 1. After heat inactivation of the enzyme at 65°C for 20 min and addition of 2.8  $\mu$ l of 10 $\times$  Ligase buffer (Fermentas), the DNA was treated with five units of T4 DNA ligase at room temperature overnight. A 1- $\mu$ l sample from the ligation mix was amplified with insert-specific primers SA130 and SA133. The PCR product was sequenced using the same primers, and both *marinerT7*-pneumococcus junctions were determined by comparison with the R6 genome to locate the position of the insert. Each insert was mapped to a unique location, where it was accompanied by a TA target duplication.

**Transformation.** Transformation of Rx derivatives was done by growing a 10-ml culture of CP1250 in CAT (19) plus 10 mM HCl to an OD<sub>550</sub> of 0.06 and inducing development of competence with CaCl<sub>2</sub> (to 0.5 mM), BSA (to 0.002%), and CSP-1 (Chiron Mimotopes, Raleigh, NC) (to 250 ng/ml). After 5 min, 1 ml of the induced cells was incubated with 100 ng of donor DNA for 70 min, diluted, and plated in CAT agar for challenge with selective antibiotic, as described previously (39). Transformation of R6, TIGR4, and their derivatives was done as described in reference 25, with the minor modifications described in reference 3.

## RESULTS

**A viable Rx mutant lacks *clpX*.** To investigate the genomic structure of strain CP1361 ( $\Delta$ *clpX::erm*), primers were designed to amplify internal fragments of *clpX* (primer pairs AP23/AP27 and AP24/AP26) or nearly the entire *clpX* gene (primer pair AP22/AP25) (Fig. 1). These primers were specifically selected so that the internal primers would amplify part of *clpX* from CP1250 (the wild-type parent strain to CP1361) but would not be expected to match sites in CP1361. In contrast, the third primer pair would create a product from CP1250 encompassing 99% of *clpX*, as well as a smaller one encompassing the *erm* insert from CP1361. Both internal *clpX* fragments and the entire *clpX* gene were readily amplified from CP1250, but no internal *clpX* fragments were obtained from CP1361 (Fig. 1). Furthermore, the size of the fragment obtained from CP1361 with AP22/AP25 was smaller than that from CP1250, in accord with the gene replacement design. We conclude that CP1361 contains an altered *clpX* gene region, as reported previously (23), but appears to lack a residual intact *clpX* gene.

**The viable Rx *clpX* deletion mutant appears to carry an unlinked suppressor of  $\Delta$ *clpX* lethality.** To seek an explanation for the discrepancy between the viability of strain CP1361 without a functional *clpX* gene and the lethality of *clpX* loss in R6, we performed a backcross that used DNA from CP1361 to transform CP1250. Em<sup>r</sup> transformants were obtained after the usual 18-h incubation of plates at 37°C, but in low yields. However, each of the five transformants analyzed carried the same molecular structure as CP1361 in the *clpX* region, as

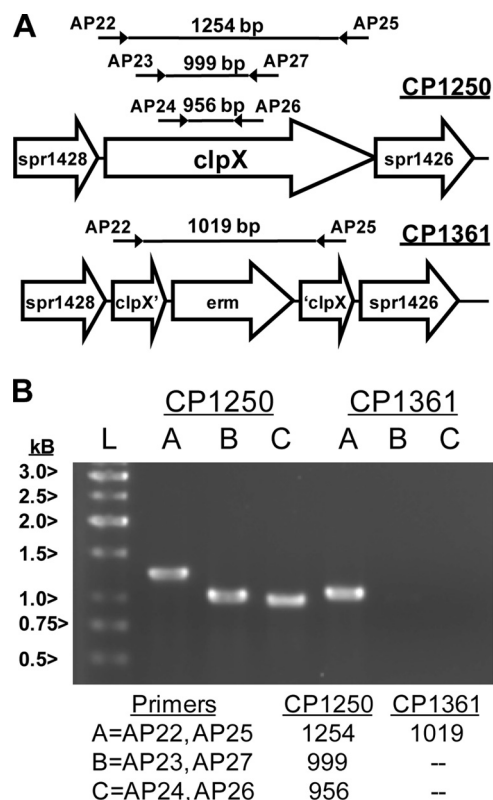


FIG. 1. Structure of an *erm* deletion/replacement mutation of *clpX* and verification of the absence of *clpX* from the mutant strain CP1361. (A) Map of ORFs in the genome of R6 and design of the *erm* insertion in CP1361. Locations of primer pairs used to amplify internal fragments of *clpX* or to reveal the size of the entire *clpX* region are indicated, as well as the sizes of predicted PCR products. ORFs in the R6 genome (accession no. AE007317) are indicated by open arrows. ' and ' indicate a truncated ORF. (B) Analysis of PCR products obtained using genomic DNA from the wild type and from CP1361 after electrophoresis on a 0.8% agarose gel. Sizes of fragments (bp) expected for the primer pairs indicated as A, B, or C, using template DNA from each strain are listed at the bottom. Sizes of reference DNA fragments (lane L) are indicated at the left.

verified by PCR, and PCR failed to recover internal fragments of the *clpX* gene (data not shown). To determine whether CP1361 might carry an unlinked extragenic suppressor of  $\Delta$ *clpX* lethality that could explain this pattern, the dose-response pattern for the CP1361  $\times$  CP1250 backcross was determined. As described by Kent and Hotchkiss (17), cotransformation by two unlinked markers in pneumococcus displays a quadratic dependence on the dose of donor DNA, while transfer of a single marker is more efficient and follows linear dependence on donor DNA dose. Production of Em<sup>r</sup> transformants followed quadratic kinetics in the backcross, while a point marker (*novo-r*) transformed the same culture of competent cells at a higher rate and with linear kinetics (Fig. 2). Thus, formation of viable Em<sup>r</sup> transformants required uptake and integration of two different fragments of donor chromosomal DNA, suggesting that CP1361 does carry both the (potentially lethal)  $\Delta$ *clpX::erm* mutation and an unlinked suppressor of the lethal effect of loss of ClpX (Fig. 2).

To verify the presence of a hypothetical unlinked suppressor

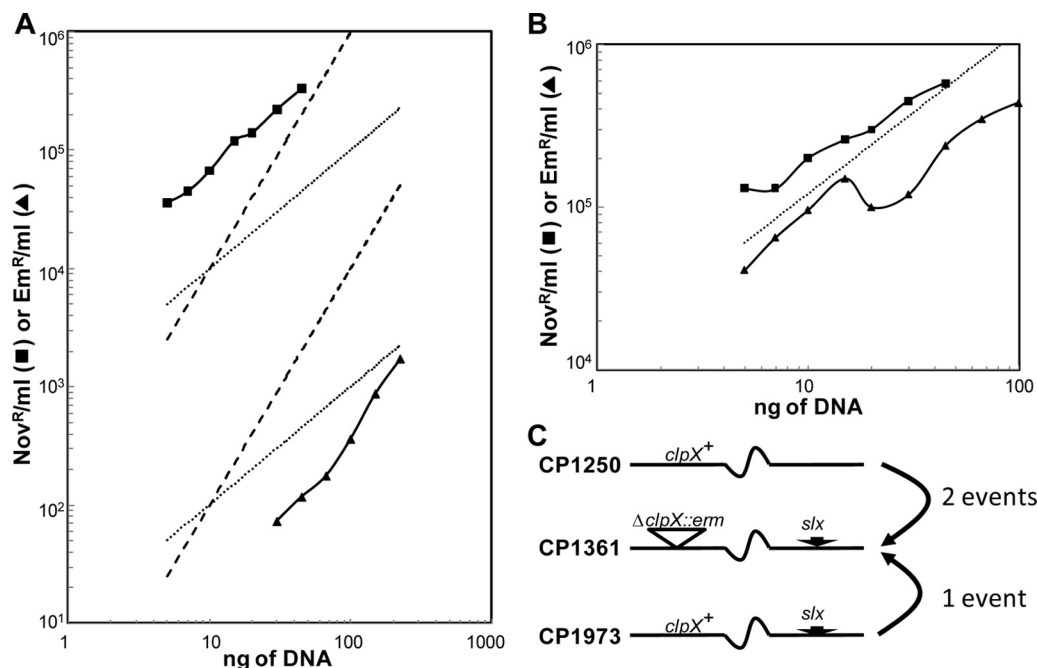


FIG. 2. Role of an unlinked suppressor in the low efficiency and two-hit kinetics of transformation of the wild type to  $\Delta clpX::erm$ . (A) Transformation of wild-type CP1250 with the indicated amounts of CP1500 genomic DNA carrying a Nov<sup>r</sup> marker (—■—) or of genomic DNA from Em<sup>r</sup> strain CP1361 (—▲—). Theoretical slopes are shown for linear (dotted;  $y = ax$ , for  $a = 10$  or 1,000) and quadratic (dashed;  $y = bx^2$ , for  $b = 1$  or 100) kinetics. (B) Transformation of the ClpX<sup>+</sup> Em<sup>r</sup> strain CP1973, using the same donor DNAs as those represented in panel A. Theoretical slope is shown for linear kinetics (dotted;  $y = 12,000x$ ). (C) Interpretation of transformation kinetics. The hypothetical suppressor of lethality of  $\Delta clpX$  (*slx*) is sufficiently distant from *clpX* that it is carried on a distinct DNA fragment and transferred independently from *clpX*. CP1973, retaining the *slx* suppressor, is transformed by CP1361 DNA via a single recombination event.

sor, the  $\Delta clpX::erm$  mutation in CP1361 was restored to wild type (*clpX*<sup>+</sup>) by high-efficiency transformation with a 7,736-bp PCR fragment carrying the wild-type *clpX* gene from CP1250. Restoration of an intact *clpX* gene in one clone (CP1973) was confirmed by PCR. To determine whether the *clpX*<sup>+</sup> strain retained a suppressor of *clpX* lethality, it was transformed using the  $\Delta clpX::erm$  marker in genomic DNA from CP1361. Em<sup>r</sup> transformants were obtained with single-hit kinetics and with a large increase in efficiency (Fig. 2). We conclude that CP1361 does indeed carry an unlinked suppressor of the lethal *clpX* phenotype and that it is retained in CP1973. We designate this mutation *slx* for suppressor of lethality of *clpX* and designate the allele in CP1361 *slx-1*.

**The suppressor maps to spr1630.** To identify the hypothetical suppressor, we employed a random insertion library as a mapping tool. Transformation of CP1973 with DNA from the *marinerT7* ( $\Omega spc$ ) library described previously (2, 3), with selection for spectinomycin resistance, created a new *slx*  $\Omega spc$  mutant library, containing 150,000 independent clones (Fig. 3). To identify  $\Omega spc$  insertions in this library that are linked to the suppressor, wild-type CP1250 was cotransformed with a mixture of the 7,501-bp  $\Delta clpX::erm$  amplicon and genomic DNA from the *slx*  $\Omega spc$  library. By virtue of the lethal effect of a *clpX* deletion in the CP1250 background, only triple transformants which had acquired  $\Delta clpX::erm$ , *slx-1*, and  $\Omega spc$  would be expected to survive selection for Em and Spc. Since the transformation of three markers would be most efficient for DNA from library members in which  $\Omega spc$  and *slx-1* are carried by the same DNA fragment, we expected many of the Em<sup>r</sup> Spc<sup>r</sup>

transformants to have  $\Omega spc$  inserts located near *slx-1*. In accord with this expectation, among 40 of the resulting Em<sup>r</sup> Spc<sup>r</sup> transformants subsequently tested for linkage between  $\Omega spc$  and *slx*, 10 exhibited elevated cotransformation of Em<sup>r</sup> with Spc<sup>r</sup>, in the range of 4 to 40% (data not shown).

The five  $\Omega spc$  insertions giving the highest yield of Em<sup>r</sup> Spc<sup>r</sup> triple transformants, i.e., those most tightly linked to *slx-1*, were mapped by inverse PCR and sequencing (Fig. 3). All five mapped to a 30-kbp region (bp 1593000 to 1623000) of the R6 genome (14).

To map the suppressor more precisely within this region, six amplicons were prepared from CP1361. Each such amplicon was cotransformed with the 7,501-bp  $\Delta clpX::erm$  amplicon, as described above, to detect its *slx* content by a single-marker selection (for Em<sup>r</sup>) for the double transformation event (*slx* and  $\Delta clpX$ ). Two overlapping 6-kbp fragments exhibited the highest *slx* activity, while *slx* activity of the four others was indistinguishable from the background (Fig. 3). To test the inference that *slx-1* is within the region of overlap, a smaller fragment (1,579 bp) representing the region of overlap was prepared and assayed for *slx* activity by cotransformation in the same way. As this new fragment also had high *slx* activity (Fig. 3), we conclude that mutation *slx-1* lies in the 1,579-bp region between bp1603661 and bp1605240.

**Loss-of-function mutations in spr1630 are suppressors of  $\Delta clpX$  lethality.** To identify the specific nature of *slx-1*, the 6-kbp amplicon exhibiting the highest *slx* activity was amplified from both the wild type and CP1361, and a portion of it was sequenced. A single-base difference between the strains was

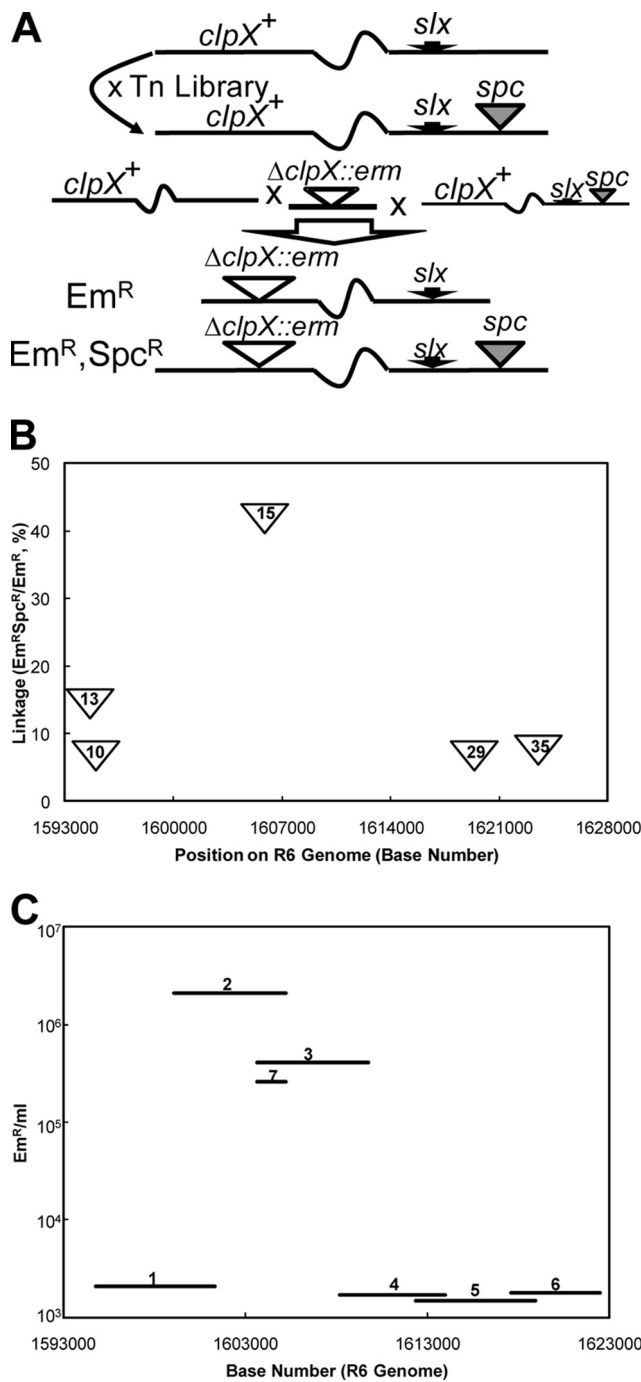


FIG. 3. Mapping of *slx* by linkage to *marinerT7* inserts. (A) Mapping strategy. Top, preparation of a *marinerT7* ( $\Omega$ *spc*) library in the *slx-1* background by transformation. Bottom, cotransfer of a  $\Delta$ *clpX::erm* amplicon, *slx-1*, and  $\Omega$ *spc* from the library. (B) Linkage of  $\Omega$ *spc* inserts to *slx*. Frequency of cotransformation of Em<sup>r</sup> and Spc<sup>r</sup> from genomic DNA of five Em<sup>r</sup> Spc<sup>r</sup> clones that had been obtained by transformation of CP1250 with *clpX::erm* and the  $\Omega$ *spc slx-1* library donor DNA. Inserts in clones 10, 13, 15, 29, and 35 were located at bp 1594927, 1594560, 1605766, 1619303, and 1623486, respectively. (C) Mapping the *slx* mutation by assay of the *slx* content of PCR products amplified from CP1361 genomic DNA. Fragments 1, 2, 3, 4, 5, 6, and 7 were amplified with primer pairs DAM850/851, DAM852/853, DAM854/855, DAM856/857, DAM858/859, DAM860/861, and DAM854/853, respectively. Bars indicate the genetic extent of each fragment and the yield of *clpX::erm* after cotransformation of CP1250 with the indicated fragment and a pure *clpX::erm* amplicon.

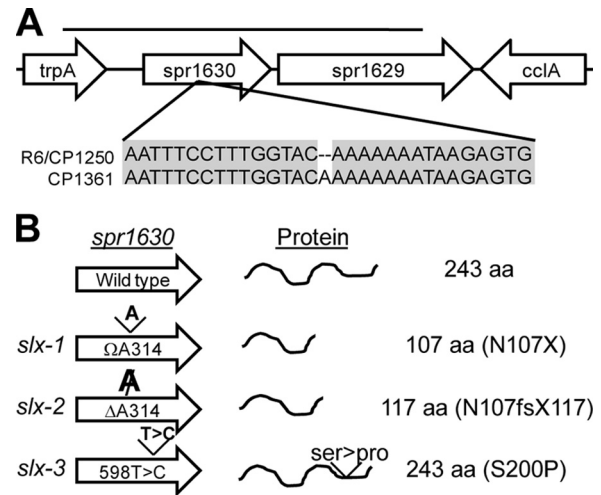


FIG. 4. Sequence changes in *spr1630* in *slx* mutants. (A) Comparison of sequences of *spr1630* in the wild type and the *slx* mutant CP1361. The sole difference found after sequencing both strands in the region indicated by the bar is shown, with identical bases shaded. (B) Sequence changes in three *slx* alleles and predicted protein products for each allele. Base changes in each allele are indicated at the left; predicted protein products are indicated at the right. fs, frameshift; X, stop codon.

found: an insertion of A, at position 1603927 on the R6 genome, which is base 314 of the predicted open reading frame (ORF) *spr1630* (Fig. 4). The mutant gene is predicted to encode a truncated protein of only 107 amino acids, suggesting that the loss of activity of *spr1630* is the cause of suppression of *clpX* lethality. We infer that the *slx-1* mutation arose spontaneously in an unstable A repeat in the course of the creation of strain CP1361, being selected because of the lethality of a *clpX* deletion in a *Slx*<sup>+</sup> background.

Indeed, the selection was so strong that it allowed us to obtain additional suppressor alleles created during PCR amplification of wild-type DNA by cotransforming with the 7,501-bp  $\Delta$ *clpX::erm* amplicon, as described above. Em<sup>r</sup> transformants were obtained by using this donor DNA, at a frequency 10-fold lower than that when using the same region amplified from CP1361, both with the 6,166-bp fragment (DAM852/853) and the 6,073-bp overlapping fragment (DAM854/855) (data not shown). Sequencing of the gene *spr1630* in three transformants recovered from transformation by the 6,166-bp fragment revealed two new mutations in *spr1630* and an independent creation of the original insertion of A314 found in CP1361. Two mutations (an insertion and a deletion) encode truncated proteins, while the third causes a replacement of a serine residue by proline (Fig. 4).

The ready recovery of *slx* mutations in *spr1630*, compared to the failure to obtain *slx* mutations from fragments elsewhere in the 30-kbp neighborhood around *spr1630*, suggested that any sufficient disruption of *spr1630* might have suppressor activity. To determine if complete loss of *spr1630* would also suppress the lethality of  $\Delta$ *clpX*, a deletion/replacement of *spr1630* was constructed by directed mutagenesis (see Materials and Methods). As this deletion also raised the transforming efficiency of the  $\Delta$ *clpX::erm* marker 100-fold (Table 3), we conclude that in the Rx background the presence of a func-

TABLE 3. Transformation efficiency of  $\Delta clpX::erm$  in *spr1630* mutants in three genetic backgrounds<sup>a</sup>

Recipient	DNA donor genotype <sup>b</sup>	
	<i>novo-r</i> (Nov <sup>r</sup> /ml)	<i>slxΔclpX::erm</i> (Em <sup>r</sup> /ml)
CP1250 (wild type)	350,000	6,000
CP1973 ( <i>slx-1</i> )	430,000	640,000
CP2008 ( $\Delta spr1630$ )	480,000	620,000
R6	4,889	289
CP2087 (R6 $\Delta spr1630$ )	3,511	14,000
TIGR4	40	0.3
CP 2089 (TIGR4 $\Delta spr1630$ )	148	557

<sup>a</sup> Exposure (70 min) of cells at OD of 0.06 to 0.1  $\mu$ g DNA/ml.

<sup>b</sup> Nov<sup>r</sup> donor DNA was from CP1500, but from CP2086 for R6 and TIGR4 recipients. Em<sup>r</sup> donor DNA was from CP1361, but from CP2088 for R6 and TIGR4 recipients.

tional *spr1630* protein is lethal in the absence of functional ClpX.

To determine whether the relationship between *spr1630* and loss of ClpX function described above is strain specific, we prepared  $\Delta spr1630$  and *clpX* mutant transformants of strain R6, another widely used descendant of strain D39, the progenitor of strain Rx, as well as of the serotype 4 strain TIGR4, which is a virulent isolate. As shown in Table 3, in both of these genetic backgrounds, the *clpX*-deficient allele was transferred at very low efficiency, but this was alleviated by deletion of *spr1630*. Thus, both the lethality of  $\Delta clpX$  and its suppression by loss of *spr1630* are shared by Rx, R6, and TIGR4.

### DISCUSSION

*clpX* mutations exhibit a variety of phenotypes in different bacterial species. When the phenotype is lethality, it may present an opportunity to identify a new critical cell function, but because of the many roles proteases may play simultaneously, unraveling such a phenotype can be difficult. For example, lethality of a *clpX* mutation has been traced to cell cycle regulation in *Caulobacter* but remains unexplained for *Synechococcus*, *Streptococcus*, and *Lactococcus*. In the present case, we have traced the lethality of *clpX* mutations to a dispensable gene and thus opened the way to the study of specific roles of ClpX in pneumococcus, as illustrated by the recent identification of ClpXP as the major player in degradation of pneumococcal SsrA-tagged proteins (1).

Inspection of the >1,000 publicly available bacterial genome sequences reveals that homologs of *spr1630* occur as part of a bi-cistronic island among certain strains of several gram-positive species. *spr1630* itself is found in 15 of 22 available pneumococcal genomes, where it is located adjacent to *trpA*. The gene is strongly conserved within this species, as the 15 cases include only six distinct alleles, differing by substitutions of 1 or 2 bp. A homolog (66% similarity) occurs in two of the three sequenced strains of *Streptococcus suis* and a few more distantly related homologs occur in *Listeria monocytogenes* (59% similarity), *Lactobacillus rhamnosus* (52% similarity), and *Lactobacillus johnsonii* (52% similarity). In *Staphylococcus aureus*, *spr1630* homologs are found as three alleles that are divergent from one another in 30 to 40 bp, in 9 of 17 sequenced genomes. In R6, *spr1630* is adjacent to *spr1629*, encoding a putative

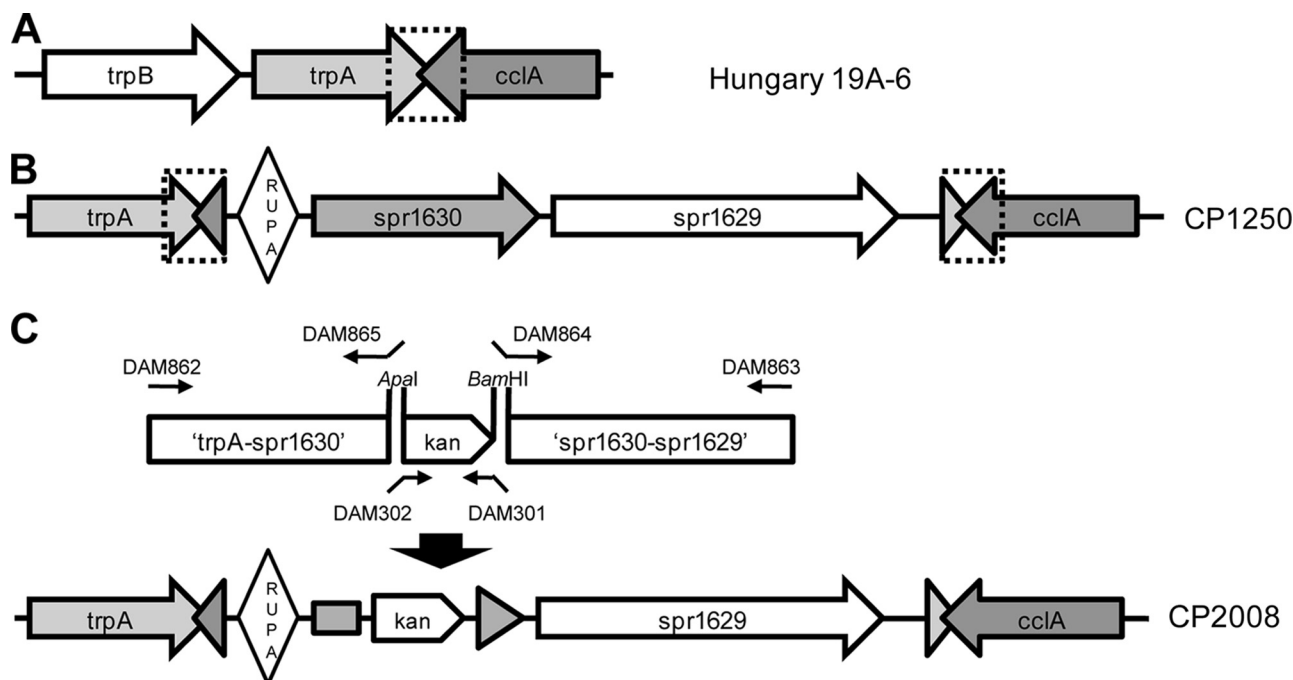


FIG. 5. Organization of the *Slx/spr1630* locus in two natural isolates and a synthetic deletion derivative. (A) Structure of the *trpA-cclA* region in Hungary19A-6 and other pneumococcal strains lacking *spr1630*. The segment duplicated in strains carrying *spr1630* is indicated by a dotted bracket. (B) Elements adjacent to *trpA* and *cclA* in the R6 genome. Diamond, RUPA; triangles, gene remnants in direct repeat. (C) Strategy for deletion of *spr1630*. PCR products ligated to *kan* to target insertion by flanking homology are depicted. Small arrows represent primers; small arrows with tails represent primers with 5' extension and restriction site.

transcriptional regulator, which contains the HTH-3 and DUF955 Pfam domains (6). Interestingly, in the other species mentioned, homologs of spr1630 are also adjacent to homologs of spr1629. In pneumococcus, the pair of genes is always found between *trpA* and *cclA*, while in *S. aureus*, it is always found adjacent to *adhE*. Thus, spr1630 appears to have been subject to horizontal gene transfer as part of a bi-cistronic operon or gene cluster, which may be self-regulating. In no case, however, has a specific function been assigned to either gene.

In pneumococcus, comparison of the genome sequence of strain R6 to those of strains that lack spr1630 alleles (including CCR11974, CDC1873, CGSP14, Hungary 19A-6, SP14-BS69, and SP23-BS72) reveals a structure that may reflect the rearrangement(s) that accompanied the original acquisition of the element (Fig. 5). spr1630 and spr1629 are immediately adjacent to a copy of a 105-bp RUPA element (31) that is otherwise found near the L11 ribosomal protein gene. The three elements are further flanked by a 107-bp direct repeat that encompasses, in spr1630-free strains, the 3' end of the *trpA* gene and the overlapping 3' end of the convergently transcribed type IV prepilin peptidase gene known as *cclA* or *cilC*. The consequence of the apparent duplication event is that the spr1630 island is isolated between two convergently transcribed operons. Since RUPA is proposed to be a vestigial insertion element occasionally activated by the transposase of IS630-SpnI, it is worth considering whether insertion of RUPA, spr1630, and spr1629 may have been accomplished in a single concerted event involving creation of the flanking duplication. This would be consistent with the proposal that RUP elements may specifically target entering foreign DNA and foster genetic rearrangements (31).

As spr1630 is quite common in pneumococcus and *Staphylococcus aureus*, it may be advantageous to both of these human pathogens and should be considered a candidate virulence factor. The report that a  $\Delta clpX$  mutant of *S. aureus* is viable (8) may suggest that the role of the spr1630 homolog in *S. aureus* or its interaction with ClpX differs significantly from that in pneumococcus; however, it is not known whether the strain in which the *clpX* mutation was evaluated carries a homolog of spr1630. While the function of spr1630 is at present entirely unknown, the special relation to ClpX implies that it acts in some critical aspect of physiology. In light of the relation to ClpX activity, it should be noted that transcription of spr1630 and spr1629 is elevated 10-fold in a *clpP* mutant (35). Because *clpP* mutants of R6, Rx, and TIGR4 are viable (but heat sensitive) the essential activity of ClpX in Slx<sup>+</sup> strains is not likely to be proteolysis. A simple hypothesis to explain its relationship to ClpX would be that the spr1630 protein constitutes a toxin, unless processed to a harmless form by a ClpX chaperone activity. Of course, the participation of ClpX may be indirect and might involve some interaction with the product of spr1629, if indeed it acts to regulate expression of spr1630.

#### ACKNOWLEDGMENT

This material is based upon work supported in part by the National Science Foundation under grant no. MCB-0543187.

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