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NOTES

Essentiality of *clpX*, but Not *clpP*, *clpL*, *clpC*, or *clpE*, in *Streptococcus pneumoniae* R6

Gregory T. Robertson,[1]† Wai-Leung Ng,[1,2] Raymond Gilmour,[1] and Malcolm E. Winkler[1,2]*

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285,[1] *and Department of Biology, Indiana University, Bloomington, Indiana 47405*[2]

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We show by using a regulated promoter that *clpX* of *Streptococcus pneumoniae* R6 is essential, whereas *clpP*, *clpL*, *clpC*, and *clpE* can be disrupted. The essentiality of *clpX* was initially missed because of duplication and rearrangement in the region of the chromosome containing *clpX*. Depletion of ClpX resulted in a rapid loss of viability without overt changes in cell morphology. Essentiality of *clpX*, but not *clpP*, has not been reported previously.

We reported previously that *clpP*-mediated proteolysis plays an important role in several pneumococcal stress responses, the development of competence, and virulence (22). ClpP proteases consist of an ATPase regulatory subunit from the Hsp100 family of chaperones and the ClpP protease subunit, which contains a serine protease active site (18; reviewed in references 8, 9, 11, 21, and 24). *Streptococcus pneumoniae* contains putative orthologs of four ATPase specificity factors (ClpC, ClpE, ClpL, and ClpX) (14, 28) and a single *clpP* gene that is in a monocistronic operon (22). *clpP* mutants showed temperature sensitivity for growth at 40°C on Trypticase soy agar blood (TSA-BA) plates but were not appreciably impaired for growth at 37°C (22). This observation was made independently by Chastanet and coworkers, who further showed that *clpE* mutants are temperature sensitive, suggesting a role for ClpE or ClpEP in temperature tolerance (5). Roles for ClpL and ClpX in *S. pneumoniae* have not yet been discovered, while the function of the ClpC specificity factor is at present in question (4, 5, 22).

We described elsewhere the construction of a Δ*clpX*::*ermAM* mutation (erythromycin resistance; Erm^r) in *S. pneumoniae* R6 (strain EL873; Table 1) and found that this mutant did not show temperature-sensitive growth like the Δ*clpP* mutant (22). However, we also reported that, for reasons that we did not understand, we could not move this Δ*clpX*::*ermAM* amplicon into *S. pneumoniae* D39 (Table 1) by transformation (22) and that we could transfer the Δ*clpX*::*ermAM* mutation from R6 to D39 only by using chromosomal DNA isolated from strain EL873 (22). Further analyses of this problem have shown that *clpX* is actually essential in *S. pneumoniae* R6 and that the *clpX* region in the chromosome forms duplications and other rearrangements at low frequency. *clpX* was required at all temperatures tested, and depletion of ClpX resulted in rapid loss of cellular viability.

Duplications and rearrangements in the *clpX* region of the *S. pneumoniae* R6 chromosome. The putative ClpX polypeptide of *S. pneumoniae* R6 shows high identity and similarity over its entire length to ClpX subunits defined for other bacteria (e.g., 58 and 76, 60 and 76, 65 and 81, and 75 and 87% identity and similarity, respectively, to ClpX of *Escherichia coli* K-12, *Caulobacter crescentus*, *Bacillus subtilis*, and *Lactococcus lactis*, respectively, in GenBank comparisons). The intercistronic regions that separate *clpX* from surrounding genes are minimal, and there are no obvious factor-independent transcription terminators in this region (Fig. 1A). Thus, *clpX* may be in a multifunctional operon that extends from *dpr* through the hypothetical gene *spr1422* (Fig. 1A). The open reading frame immediately downstream from *clpX* designated *spr1426* was recently shown to be essential and may encode a GTP binding protein (29). The *dfr* gene upstream from *clpX* (Fig. 1A) encodes dihydrofolate reductase, which is required for growth of a number of bacterial species (12). The other known gene upstream of *clpX* is *dpr*, which has been implicated in H_2O_2 resistance (33) and may be critical for growth of *S. pneumoniae*, which produces H_2O_2 during aerobic growth under laboratory conditions (26).

Previously, we confirmed the presence of the Δ*clpX*::*ermAM* allele in EL873 by PCR with primers located immediately outside the *clpX* reading frame (22). Although we thought that we had sufficient resolution on gels to resolve the Δ*clpX*::*ermAM* construct from the $clpX^+$ gene, later analyses and further digestion of the PCR products with restriction enzymes revealed that copies of both the Δ*clpX*::*ermAM* and $clpX^+$ genes were present in EL873 (Fig. 2, lane 5). Thus, EL873 is a merodiploid that most likely arose by recombination of the Δ*clpX*::*ermAM* allele into one copy of a duplication of the *clpX* region that occurred spontaneously in the chromosome of *S.*

* Corresponding author. Mailing address: Department of Biology, Indiana University, Jordan Hall 142, Bloomington, IN 47405. Phone: (317) 508-7812. Fax: (812) 855-6705. E-mail: mwinkler@bio.indiana.edu.

† Present address: Cumbre, Inc., Dallas, TX 75235.

TABLE 1. Bacterial strains[a]

Strain	Genotype or phenotype	Description	Source or reference
EL59	*S. pneumoniae* R6, avirulent unencapsulated parent strain	Derived from D39 isolate	A. Tomasz (14)
EL161	*S. pneumoniae* D39, virulent encapsulated type 2 parent strain	Subclone of original clinical isolate	J. Yother (25)
EL539	EL59 Δ*clpP*::*aad9* (Spc^r)	EL59 transformed with linear Δ*clpP*::*aad9* amplicon	22
EL854	EL59 Δ*clpC*::*ermAM* (Erm^r)	EL59 transformed with linear Δ*clpC*::*ermAM* amplicon	22
EL873	EL59 Δ*clpX*::*ermAM clpX*$^+$ (Erm^r)	EL59 containing previously undetected merodiploid of Δ*clpX*::*ermAM* and *clpX*$^+$	22
EL1039	EL161 Δ*clpX*::*ermAM clpX*$^+$ (Erm^r)	EL161 transformed with genomic DNA from EL873; contains a previously undetected merodiploid of Δ*clpX*::*ermAM* and *clpX*$^+$	22
EL1082	EL59 Δ*clpL*::*ermAM* (Erm^r)	EL59 transformed with linear Δ*clpL*::*ermAM* amplicon	This study
EL1259	EL59 Δ*clpE*::*aad9* (Spc^r)	EL59 transformed with linear Δ*clpE*::*aad9* amplicon	This study
EL1383	EL59 Δ*bgaA*::(P$_c$::*tetL*::T1T2::P$_{fcsK}$-*clpX*$^+$) (Tet^r)	EL59 transformed with linear Δ*bgaA*::(P$_c$::*tetL*::T1T2::P$_{fcsK}$-*clpX*$^+$) amplicon	This study
EL1387	EL1383 Δ*clpX*<>*ermAM* (Erm^r Tet^r)	EL1383 transformed with linear Δ*clpX*<>*ermAM* amplicon	This study
EL1392	Δ*clpX*<>*ermAM clpX*$^+$ (Erm^r)	EL59 transformed with Δ*clpX*<>*ermAM*, resulting in a partially characterized duplication of Δ*clpX*<>*ermAM* and *clpX*$^+$	This study

[a] Strains were constructed by transformation of indicated recipients with linear double-stranded synthetic PCR amplicon DNA. Following single colony isolation, all constructions were confirmed by PCR analysis of chromosomal DNA by using flanking and internal primers and additional restriction digestion of amplicons (details available at http://www.streppneumoniae.com).

pneumoniae R6 (e.g., see reference 2). This conclusion was fully supported by the observation that EL873 reverted rapidly to sensitivity to erythromycin when antibiotic selection was removed from cultures (Table 2), and this reversion was accompanied by restoration of wild-type *clpX*$^+$ in the chromosome (Fig. 2, lanes 5 and 6).

Because of the potential for polarity of *clpX* insertions on expression of the essential downstream *spr1426* gene (Fig. 1A), we attempted to cross the Δ*clpX*<>*ermAM* amplicon depicted in Fig. 1A, which has the *clpX* reading frame replaced exactly from its start to stop codon by the *ermAM* reading frame, into the chromosome of *S. pneumoniae* R6 by transformation induced by synthetic competence stimulatory peptide 1 (see reference 13). The frequency of transformation of this amplicon (<2.0×10^{-6} Δ*clpX*<>*ermA* transformants) was below the level of detection compared with that of control genomic DNA imparting resistance to novobiocin (7.1×10^{-4} Nor^r transformants) in R6 parent strain EL59. The frequency of transformation was calculated based on the number of recovered antibiotic-resistant colonies from 1 ml of transformation mixture divided by the total CFU per milliliter. However, one Erm^r colony did appear (EL1392; Table 1), which contained another kind of rearrangement in the *clpX* region. Analysis of the *clpX* region from EL1392 by PCR showed the presence of an anom-

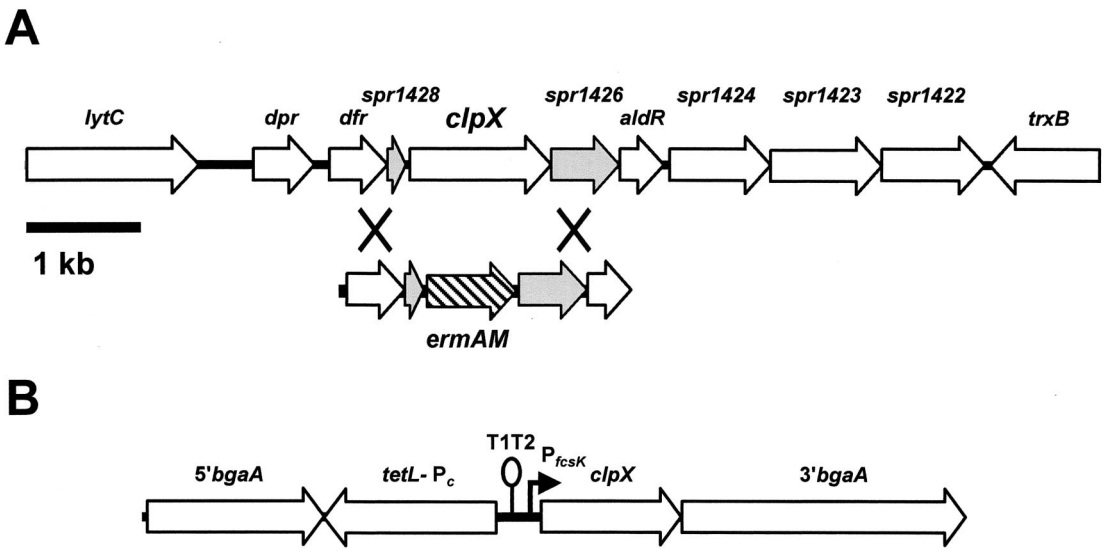

FIG. 1. Organization of the *clpX* operon in *S. pneumoniae* R6 and construction of a nonpolar replacement of the *clpX* reading frame with *ermAM* (Erm^r) (A) in a strain containing an ectopic copy of *clpX*$^+$ under the control of the fucose-inducible P$_{fcsK}$ promoter located in the *bgaA* locus (B) (see text and Table 1 for details). Genes and predicted directions of transcription are depicted by arrows (drawn to scale). The *lytC* and *trxB* genes likely mark the boundaries of the *clpX* region based on the large intergenic region and the direction of transcription, respectively. The shaded regions depict the flanking genes surrounding *clpX*, and the hatched region corresponds to the *ermAM* open reading frame. P$_c$-*tetL* imparts constitutive resistance to 0.25 μg of tetracycline per ml and was used as a selection marker. T1T2 indicates transcription terminators to protect regulated expression from P$_{fcsK}$.

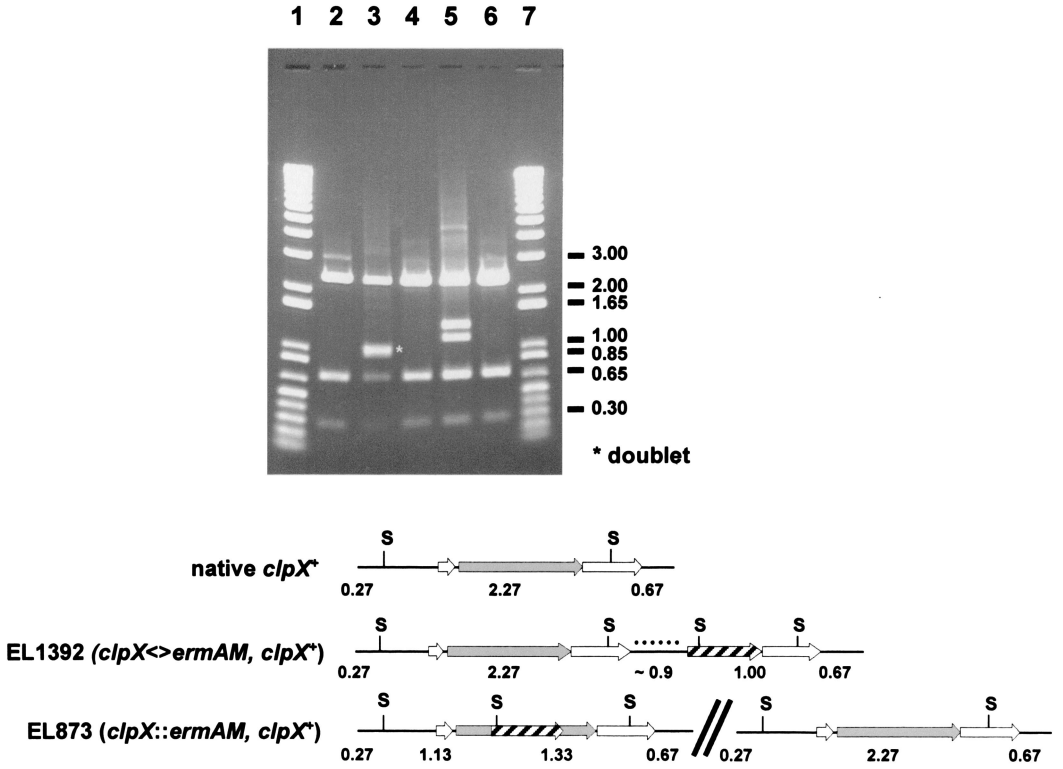

FIG. 2. *Sca*I restriction maps of the *clpX* regions of genomic DNA of *S. pneumoniae* strains EL59, EL873, and EL1392 amplified by PCR with the oligonucleotide primer pair clpX-a and clpX-c (22). Lanes 1 and 7, Gibco 1-kb Plus standard ladder; lane 2, wild-type $clpX^+$ locus from parent strain EL59; lane 3, duplicated *spr1426* locus from EL1392, which contains a copy of $clpX^+$ and the *clpX<>ermAM* cassette; lane 4, Erm^s revertant of EL1392 containing the wild-type $clpX^+$ region; lane 5, *clpX::ermAM* with duplicated $clpX^+$ locus from EL873; lane 6, Erm^s revertant of EL873 containing the wild-type $clpX^+$ region. Shaded arrows, *clpX* reading frame; hatched arrows, *ermAM* reading frame; open arrows, genes surrounding *clpX* (Fig. 1); black dots, ~0.9 kb of unknown DNA present in EL1392 $clpX^+$ region. *Sca*I restriction sites are indicated with the letter S and a horizontal line. The break denotes an unknown amount of chromosomal DNA in the EL873 merodiploid. Predicted restriction fragment sizes and indicated DNA standards are in kilobase pairs.

alous ~5-kb amplicon that contained the *ermAM* cassette, $clpX^+$, a duplication of the flanking downstream gene *spr1426*, and an uncharacterized region of ~0.9 kb containing DNA of unknown origin (Fig. 2, lane 3). This arrangement probably arose by an aberrant recombination event in the *clpX* region. Unlike EL873, reversion of EL1392 to erythromycin sensitivity occurred at a very low frequency when antibiotic selection was removed from cultures (Table 2), and again, reversion was accompanied by restoration of wild-type $clpX^+$ in the chromosome (Fig. 2, lanes 3 and 4).

We determined the frequency at which Δ*clpX<>ermAM* $clpX^+$ merodiploids arose in transformations compared to that for the unlinked essential *gyrB* gene (29). Ten independent exponential cultures of EL59 (R6 parent strain) in brain heart infusion (BHI) medium were diluted 1:20 in 1 ml of BHI medium containing 10% heat-inactivated horse serum (Sigma), 10 mM glucose, and 100 ng of competence stimulatory peptide 1 to give a cell density of $\sim 7.0 \times 10^5$ CFU/ml (22). Competent cell suspensions were then mixed with the nonpolar Δ*clpX<>ermAM* amplicon, a Δ*gyrB::ermAM* deletion-insertion amplicon, or control genomic DNA bearing a point mutation which confers resistance to novobiocin (Nov^r) (16). Nov^r transformants were recovered from these transformations at an average frequency of $\sim 6 \times 10^{-4}$, whereas Δ*clpX<>ermAM* or Δ*gyrB::ermAM* transformants appeared at a significantly lower frequency of $\leq 10^{-6}$. Thus, formation of Δ*clpX<>ermAM* $clpX^+$ duplications occurred at a low detectable frequency comparable to that for other essential genes, such as Δ*gyrB::ermAM* $gyrB^+$, elsewhere in the chromosome of *S. pneumoniae* R6.

TABLE 2. Reversion frequency of *S. pneumoniae* mutants containing duplications in the *clpX* region of the chromosome

Strain	Selection during growth[a]	CFU/ml[b]	No. of colonies screened	No. (%) of Erm^r and Erm^s isolates following spontaneous resolution of duplications	
				Erm^r	Erm^s
EL873	None	4.7×10^6	31	4 (13)	27 (87)
	ERY	4.9×10^5	31	31 (100)	0 (0)
EL1392	None	2.0×10^7	130	129 (99.2)	1 (0.8)
	ERY	7.9×10^6	61	61 (100)	0 (0)

[a] Bacteria were grown statically in BHI containing or lacking 0.3 μg of erythromycin (ERY) per ml for ≈7 h at 37°C in 5% CO_2, serially diluted in 0.9% (wt/vol) NaCl, and spread onto TSA-BA plates containing or lacking 0.3 μg of erythromycin per ml, respectively. Random colonies were screened for erythromycin resistance (Erm^r) or sensitivity (Erm^s) by being patched onto TSA-BA plates containing or lacking 0.3 μg of erythromycin per ml.

[b] Number of CFU per milliliter of culture from which cells were recovered at the time of plating.

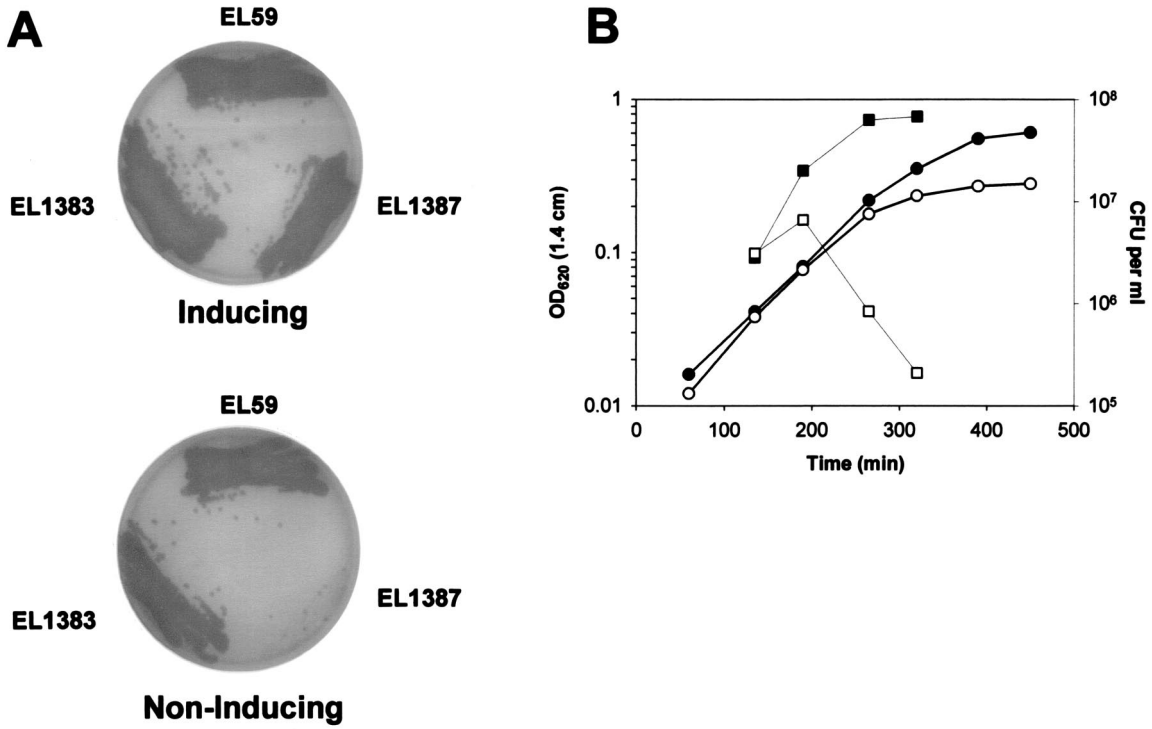

FIG. 3. Impaired growth of *S. pneumoniae* R6 strain EL1387 containing a regulated replacement of *clpX* (Δ*clpX* P_{fcsK}::*clpX*$^+$) on medium lacking fucose. (A) EL59 (*clpX*$^+$ parent), EL1383 (regulated merodiploid *clpX*$^+$ P_{fcsK}::*clpX*$^+$), and EL1387 (regulated replacement Δ*clpX* P_{fcsK}::*clpX*$^+$) were streaked onto TSA-BA plates containing 0.25% (wt/vol) fucose (inducing) or lacking additional fucose (noninducing). Plates were photographed after 24 h of incubation at 37°C in an atmosphere of 5% CO_2. (B) EL1387 (regulated replacement Δ*clpX* P_{fcsK}::*clpX*$^+$) was grown statically overnight in BHI broth containing 0.1% (wt/vol) fucose at 37°C in 5% CO_2. Cultures were then diluted 100-fold into fresh BHI containing or lacking 0.1% (wt/vol) fucose, and static incubation was continued at 37°C in 5% CO_2. Filled circles and squares represent the optical densities and viable cell counts, respectively, of the culture containing 0.1% (wt/vol) fucose. Open circles and squares represent the optical densities and viable cell counts, respectively, of the culture lacking fucose (0.001% [wt/vol]) carryover fucose from starting culture). Results are representative of at least two independent experiments. OD_{620} (1.4 cm), optical density at 620 nm for a tube with a 1.4-cm diameter.

***clpX* is essential in *S. pneumoniae* R6.** The fact that we could not obtain a simple transformant that replaced *clpX* with Δ*clpX*<>*ermAM* suggested that *clpX* was essential. To demonstrate essentiality, we needed to construct a merodiploid that put *clpX*$^+$ transcription under the control of a regulatable promoter. Several catabolic promoters have been shown elsewhere to be regulated by sugars in *S. pneumoniae* and related species, including those for galactose, maltose, and fucose utilization (1, 3, 3a, 20; P. F. Chan, K. A. Ingraham, C. Y. So, M. Lonetto, M. Rosenberg, D. J. Holmes, and M. Zalacain, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. H-97, p. 370, 2000). We chose the promoter upstream from the fucose kinase gene (*fcsK spr1973*) (P_{fcsK}) (14), because it had previously been reported to provide regulation in *S. pneumoniae* (3a; W. Bae, P. Chan, L. Palmer, V. Clausen, J. Throup, M. Noordewier, K. Koretke, D. Lunsford, A. Bryant, K. Ingraham, D. Holmes, M. Rosenberg, and M. Burnham, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. B-381, p. 126, 2001). We constructed the cassette shown in Fig. 1B in which the promoter region of *clpX*$^+$ was replaced by P_{fcsK}, which is protected by transcription terminators T1T2 and connected to a *tetL* (tetracycline resistance [Tet^r]) gene driven from a constitutive promoter (P_c) (16). The P_c-*tetL*::T1T2::P_{fcsK}-*clpX*$^+$ cassette was crossed into the *bgaA* locus, which encodes a dispensable β-galactosidase (34) that was partly deleted in the construction, to yield strain EL1383 (Table 1). Tet^r transformants were selected on TSA-BA plates containing 0.25% (wt/vol) L-fucose at 37°C in an atmosphere of 5% CO_2. The location of the construction was confirmed by PCR (data not shown).

The Δ*clpX*<>*ermAM* amplicon was then crossed into the chromosome of EL1383, and Erm^r mutants were selected on TSA-BA plates containing the inducer 0.25% (wt/vol) L-fucose. Now the frequency of recovery of Erm^r transformants increased dramatically (2.1×10^{-3} Δ*clpX*<>*ermA* transformants compared to $<2.0 \times 10^{-6}$ in EL59 [see above]) and was comparable to that for the Nov^r control (4.5×10^{-4}). The resulting regulated replacement EL1387 (Table 1) grew like EL59 (*clpX*$^+$ parent) and EL1383 [Δ*bgaA*::(P_c-*tetL*::T1T2::P_{fcsK}-*clpX*$^+$) *clpX*$^+$ merodiploid] on plates containing L-fucose but completely failed to grow when L-fucose was omitted from plates (Fig. 3A). The lack of growth of EL1387 in the absence of L-fucose is not consistent with a polar effect on expression of the essential downstream *spr1426* gene, because only expression of the *clpX*$^+$ gene was under the control of L-fucose in the Δ*bgaA* locus. Thus, expression of *clpX*$^+$ is essential for the growth of *S. pneumoniae* R6.

Although *clpX* is essential and *clpP* is not, it is formally possible that the essentiality of *clpX* could depend on a functional *clpP*$^+$, *clpC*$^+$, *clpE*$^+$, or *clpL*$^+$ gene. For example, ClpX

could interact with the ClpP protease subunit or one of the other ATPase specificity subunits to limit ClpP proteolysis activity. To test this notion, we transformed EL1387 grown in fucose-containing medium with a *clpP*::*kan*::*rpsL*$^+$, *clpC*::*kan*::*rpsL*$^+$, *clpE*::*kan*::*rpsL*$^+$, or *clpL*::*kan*::*rpsL*$^+$ Janus cassette amplicon (27). For each transformation, we recovered hundreds of transformants on TSA-BA plates containing fucose and kanamycin but no transformants on TSA-BA plates containing kanamycin but lacking fucose (data not shown). Thus, *clpX* essentiality did not depend on a functional *clpP*$^+$, *clpC*$^+$, *clpE*$^+$, or *clpL*$^+$ gene.

Phenotypes of *clpX* underexpression. We examined phenotypes of bacteria depleted for ClpX to learn more about the basis for the essentiality of *clpX*. Repetition of the experiment shown in Fig. 3A at 30°C showed that *clpX* expression was essential at the lower temperature as well as at 37°C for bacteria spread onto TSA-BA plates (data not shown). We found that 0.05% (wt/vol) fucose was the minimum concentration that supported growth of regulated replacement mutant EL1387 in BHI broth at 37°C; however, this concentration of fucose led to lower growth yields of EL1387 than of the regulated merodiploid EL1383 (final culture optical density at 620 nm, 0.17 compared to 0.47, respectively). We next determined the viability of cells of the regulated replacement mutant EL1387 following downshift to a low fucose concentration (Fig. 3B). Addition of 0.1% (wt/vol) fucose supported full growth of viable cells in BHI broth at 37°C (Fig. 3B, closed symbols). In contrast, cultures of EL1387 downshifted to 0.001% (wt/vol) fucose prematurely stopped increasing in optical density and concomitantly rapidly lost cellular viability (Fig. 3B). The plateau in optical density suggested that EL1387 was not undergoing rapid autolysis. Consistent with this observation, examination of EL1387 by phase-contrast microscopy at various times after fucose downshift did not reveal significant changes in cellular morphology compared to that of EL59 and EL1383 controls (data not shown). At 360 min after fucose downshift, <1% of the EL1387 cells were irregularly shaped and large, especially at the end of short chains, compared with those of the EL59 or EL1383 controls (data not shown); however, at this point significant death was occurring in the EL1387 cultures.

The rapid cell death of EL1387 upon ClpX depletion (Fig. 3B) made analysis of global transcription patterns by microarrays (19, 22) problematic. Instead we tested whether potential overexpression of ClpX in the regulated merodiploid EL1383 changed the transcription pattern. We compared microarray patterns of RNA isolated from cultures of EL1383 grown to an optical density at 620 nm of ~0.2 in BHI at 37°C lacking or induced for 60 min with 0.2% (wt/vol) fucose. On the basis of two independent experiments, we were unable to detect significant differences in transcript patterns of EL1383 cultured with and without fucose, with the exception of the *clpX* transcript (increased ~1.7-fold) and those of genes *spr1963* to *spr1973*, which comprise the fucose regulon (*fcsK*, *fucA*, *fucU*, PTS-EII [*spr1970*], PTS-EII [*spr1969*], PTS-EII [*spr1968*], PTS-EII [*spr1967*], *spr1966*, *spr1965*, *fucI*, and *adh2* increased ~23-, 16-, 15-, 11-, 39-, 25-, 10-, 10-, 36-, 28-, and 3-fold, respectively). It is possible that overexpression of *clpX* was not sufficient in these experiments to elicit a phenotype.

Implications. Our finding that *clpX* is essential, but *clpP* is not, is unusual, because ClpX usually functions as regulatory subunit for a ClpXP protease (10, 32). We constructed and confirmed the presence of single-copy insertion-deletion mutations that inactivate *clpP*, *clpC*, *clpL*, or *clpE* (strain EL539, EL854, EL1082, or EL1259, respectively [Table 1]). None was essential for growth of *S. pneumoniae* R6 at 37°C (data not shown), and only *clpP* and *clpE* imparted temperature sensitivity in BHI liquid medium as reported previously (5, 22; data not shown). Curiously, the *clpE* mutant was not temperature sensitive on TSA-BA plates at 40°C (data not shown), whereas the *clpP* mutant was (22). The cyanobacterium *Synechococcus* sp. strain PCC 7942 has genes encoding three ClpP isoenzymes and a single copy of ClpX. Viable deletion-insertion mutants of *Synechococcus* were obtained only for *clpPI* and *clpPII*, suggesting that *clpPIII* and *clpX* are indispensable for growth (6, 23). ClpX and ClpP are both essential in *C. crescentus* (7, 15), and chaperone functions have been ascribed to ClpX besides its role in ClpXP proteolysis (17, 24, 30). A recent report shows that *E. coli* K-12 lacking functional ClpX or ClpP lost viability more rapidly than did wild-type parent strains during extended stationary phase (31).

The results reported here indicate that ClpX must play essential roles independent of ClpP in *S. pneumoniae* R6 (Fig. 3A; also see above). Furthermore, depletion of ClpX leads to rapid cell death without overtly affecting cell morphology (Fig. 3B; data not shown). Unlike *clpP*, *clpC*, *clpE*, and *clpL*, *clpX* appears not to be a member of the heat shock regulon in *S. pneumoniae* (22), and microarray analyses indicated that the *clpX* transcript amount was not significantly changed by phase of growth in chemically defined medium or by sublethal concentrations of the antibiotic triclosan or novobiocin or most common translation inhibitors, except for the macrolides erythromycin and roxithromycin, which caused marginal 1.7-fold induction of *clpX* transcript amounts (19; data not shown). Genetic and physiological experiments are in progress to learn the essential function(s) of ClpX and to understand the organization and possible regulation of the *clpX* operon (Fig. 1) in *S. pneumoniae*.

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