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Evolution

Chimeric phage–bacterial enzymes: A clue to the modular evolution of genes

(autolysin/lysozyme/protein engineering/pneumococcus/phages)

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ABSTRACT **Pneumococcal peptidoglycan amidase (*N*-acetylmuramoyl-L-alanine amidase, EC 3.5.1.28) and phage CPL1 lysozyme degrade a common substrate (choline-containing pneumococcal cell walls); the former hydrolyzes the bond between muramic acid and alanine, whereas the latter breaks down the linkage between muramic acid and glucosamine. The amino acid sequences of their C-terminal domains are homologous. Chimeric genes were constructed by site-directed mutagenesis: a unique *Sna*BI restriction site in the *cpl1* gene, coding for the phage lysozyme, was introduced at a location equivalent to the *Sna*BI site present in the *lytA* gene, which codes for the pneumococcal amidase. The resulting genes expressed lytic activities at levels similar to those of the parental genes. The gene products, which have been purified to electrophoretical homogeneity, exhibited unusual combined biochemical properties—e.g., by exchange of protein domains, we have switched the regulatory properties of these enzymes without altering their catalytic activities. Chimeric gene construction in *Streptococcus pneumoniae* and its bacteriophages is an excellent model to study the modular organization of genes and proteins and to help to establish evolutionary relationships between phage and bacteria. These constructions provide an experimental approach to the molecular processes involved in cassette recruitment during evolution and contribute support to the concept of bacteria as adaptable chimeras.**

The modular organization of genes and proteins has been repeatedly suggested as an evolutionary principle (1). This theory has received strong support from the comparison of sequences available in the data banks (2). Unfortunately, there is only a limited number of direct experimental approaches backing this hypothesis, mostly dealing with the construction of new chimeric proteins by fusing cloned genes (3–5). These genetic manipulations have opened new insights into studies of the relationships between molecular structure and the biological function of proteins—e.g., it has been demonstrated that reconstruction of an enzyme by domain substitution between two proteins that share extensive amino acid sequence similarity resulted in the switch of substrate or catalytic specificity (6–8).

We also have suggested the idea of modular enzyme organization of the peptidoglycan lytic enzymes of *Streptococcus pneumoniae* and its bacteriophages on the basis of their structural and functional relationships (9). *S. pneumoniae* contains two autolysins that hydrolyze covalent bonds of the cell wall, a powerful *N*-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28) and an *N*-acetylglucosaminidase (10). The amidase is the agent responsible for the separation of daughter cells at the end of cell division (11) and also seems to contribute to virulence of this species (12). In addition, this enzyme participates in the liberation of progeny bacteriophage into the medium (13). The pneumococcal amidase is encoded by the *lytA* gene, and we have demonstrated that the low-activity form of the enzyme (E-form) is the primary translation product of this gene (14). The fully active form of this amidase (C-form) is found only in pneumococci that contain choline in the teichoic acids of the cell wall (15), and this amino alcohol has been identified as an allosteric ligand necessary for the recognition and degradation of cell walls by the enzyme (16). The E-form also can be converted *in vitro* to the C-form by incubation either with choline-containing cell walls or with 2% free choline (17, 18). The choline-mediated "conversion" of the amidase resulted in a stable C-form of the enzyme (17, 18). Nevertheless, when the conversion was achieved by using 2% choline, this aminoalcohol must be withdrawn from the assay, either by dilution or dialysis, before we test the activity of the enzyme since this activity was inhibited by choline (0.1% and higher) in a noncompetitive manner (16, 17). The inhibition has been postulated to be due to a desorption of the enzyme from its substrate by choline (17).

On the other hand, the lytic enzymes encoded by the infecting bacteriophages are also involved in the process of phage liberation—e.g., a lysozyme (peptidoglycan *N*-acetylmuramoylhydrolase, EC 3.2.1.17) (CPL1) encoded by the *cpl1* gene of bacteriophage Cp-1 (19). CPL1 lysozyme encoded by the *cpl1* gene of the pneumococcal bacteriophage Cp-1 does not require the process of conversion to achieve full enzymatic activity, although this lysozyme shares with the pneumococcal amidase an absolute requirement for choline-containing teichoic acid for activity (19). Again, the activity of the CPL1 lysozyme was also inhibited by choline (0.01% and higher) (19).

Comparison of the primary structures of these two lytic enzymes showed remarkable identity of their C-terminal domains, since 73 of 142 amino acid residues were identical and 55 of the remaining 69 nonidentical residues were conservative substitutions. We postulated that the C-terminal domains are regulatory and are responsible for the recognition of choline-containing cell walls. In contrast, the N-terminal domains are catalytic and contain active sites of different specificities: the lysozyme hydrolyzes peptidoglycan between carbohydrate units, whereas the amidase separates the peptide and carbohydrate chains (9). Thus, we conjectured that the domains might be interchangeable, and that their recombination might create active chimeric enzymes with novel properties.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Phages. The *Escherichia coli* strains used were HB101 (20), JM83 (21), and JM103 (21). The latter was used as a host for phage M13tg130 (Amersham). The pneumococcal bacteriophage Cp-1 has been described elsewhere (22). *S. pneumoniae* M31 is a mutant showing a complete deletion of the *lytA* gene (23). Plasmids pGL80 (14), pGL81 (24), and pGL100 (24) carry the *lytA*

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gene. Plasmid pCIP50 (19) contains the *cpll* gene. Plasmid pBR325 (25) and pUC13 (21) were used in subcloning experiments. Phage M13tg130::*cpll* was obtained as described (26).

Plasmid Isolation and Transformation. Plasmid DNA was prepared as described by Birnboim and Doly (27). *E. coli* HB101 or JM83 were transformed by using the RbCl procedure as described (19).

Oligonucleotide Site-Directed Mutagenesis. The mutagenic oligonucleotide primer was synthesized by using an Applied Biosystems DNA synthesizer. Mutagenesis was achieved by using the mutagenesis system of Amersham.

DNA Sequence Analysis. DNA sequencing was performed by the dideoxy chain-termination method (28). Nucleotide sequences of *cpll* and *lytA* genes have been reported (9, 14).

Characterization of Lytic Activity. The type of enzyme activity present was determined to be either "amidase" or "lysozyme" by analysis of the degradation products of radioactively labeled cell walls as described (19). [2-^{14}C]-Ethanolamine-labeled cell walls used to test the specificity for choline recognition were prepared as described (19).

Assay for Autolytic Activity. Specific activity, K_m, and inhibition by choline were determined by using [*methyl*-^{3}H]choline-labeled cell walls as substrate (19). One unit of lytic activity was defined as the amount of enzyme that catalyzes the hydrolysis (solubilization) of 1 μg of cell wall material in 10 min (19).

Plasmid Constructions. The structural *lytA* gene contains a *Sna*BI restriction endonuclease site that corresponds to Tyr-187 of the translated polypeptide sequence (14) (Fig. 1). The structural *cpll* gene lacks the *Sna*BI site, so we introduced by site-directed mutagenesis this restriction site at a position comparable to that of the *lytA* gene (corresponding

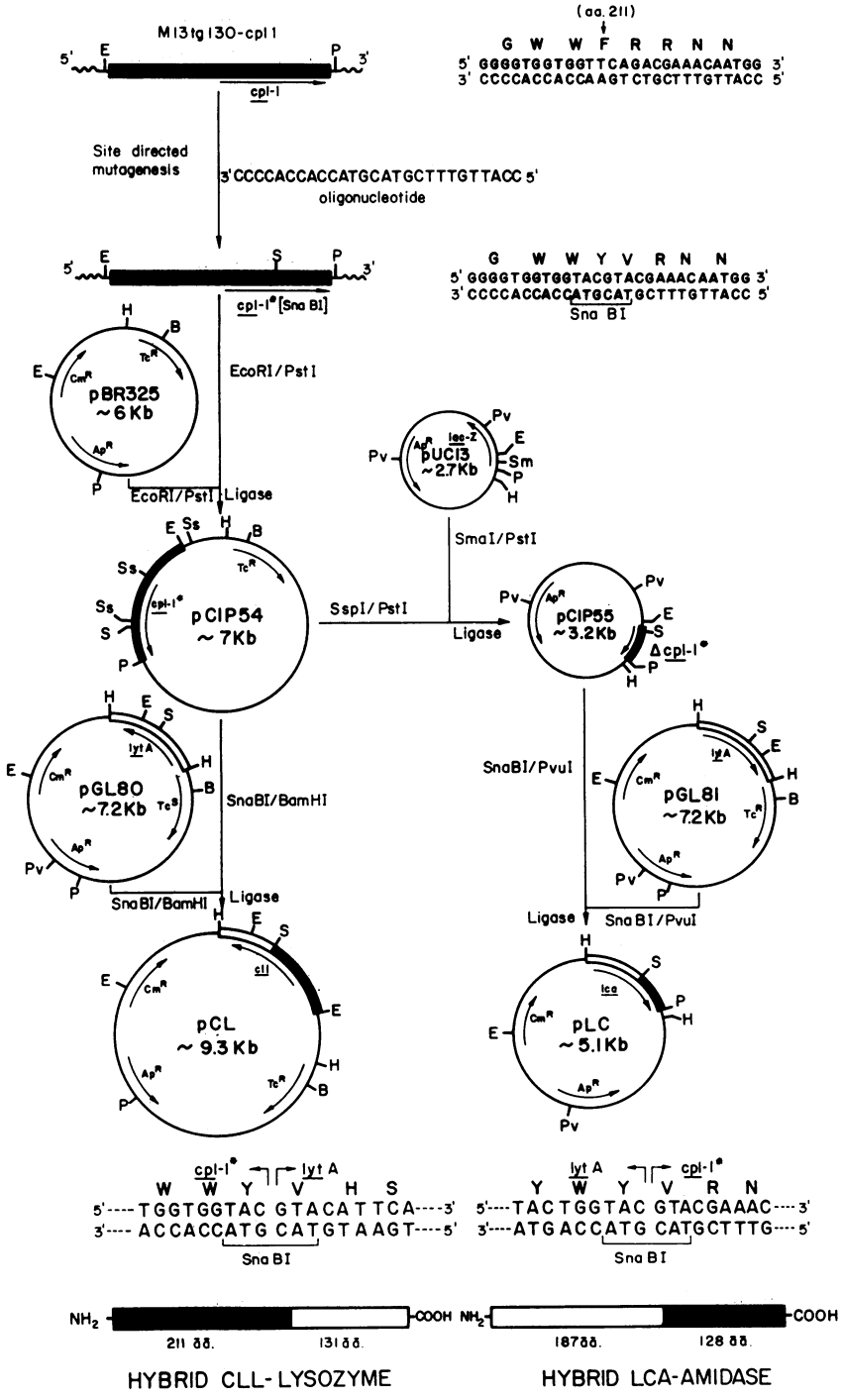

FIG. 1. Schematic representation of the construction of plasmids pCL and pLC bearing the recombinant *cpll*:*lytA*=*cll* and *lytA*:*cpll*=*lca* fusions. A *Sna*BI restriction site corresponding to the *Sna*BI site in *lytA* gene was introduced into the phage-borne *cpll* gene (M13tg130::*cpll*) by oligonucleotide site-directed mutagenesis. The upper and lower duplex sequences show the nucleotide and amino acid sequences (in one-letter code) of the region surrounding amino acid 211 of CPL1 lysozyme and of the mutated enzyme, respectively. The new *Sna*BI site of the mutated *cpll** gene is indicated in the lower duplex sequence. The sequence of the oligonucleotide used for site-directed mutagenesis is also shown. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; Pv, *Pvu* I; S, *Sna*BI; Sm, *Sma* I; Ss, *Ssp* I; Δ, a deleted gene. The cloned *lytA* and *cpll* genes are given by white and black blocks, respectively. The headed arrows indicate the direction of transcription of the genes. At the bottom of the figure we show the nucleotide and the deduced amino acid sequences surrounding the *Sna*BI site of the chimeric genes as well as the schematic representations of the new chimeric proteins. Antibiotics are indicated by: Ap, ampicillin; Cm, chloramphenicol; and Tc, tetracycline. Superscripts R and S indicate resistance or sensitivity to antibiotics, respectively.

to Phe-211 of the CPL1 lysozyme) (9) (Fig. 1). The chimeric plasmids pCL and pLC were constructed as indicated in Fig. 1. The regions surrounding the gene fusions were sequenced to confirm that the desired in-frame ligations had been achieved.

RESULTS

Construction of Chimeras. Based on the known DNA sequences of the *lytA* and *cpl1* genes, chimeric proteins have been constructed (Fig. 1). The alignment of the sequences of these enzymes allowed us to identify possibly equivalent points on the two genes (9). The new proteins were generated through *in vitro* recombination of DNA at equivalent positions on the *lytA* and the *cpl1* genes as defined by the alignment. We carried out this substitution by fusing the two genes, *lytA* and *cpl1*, at sites approximating the junction zone of the N- and C-terminal domains of these enzymes (9).

As shown above (see *Materials and Methods*), we created a single *Sna*BI site in the *cpl1* gene to facilitate the chimeric constructions. We verified that the introduction of the *Sna*BI site was neutral with respect to the reading frame of the gene and to the enzymatic activity—i.e., in spite of the amino acid changes (Phe → Arg by Tyr → Val) that the mutation generated in the CPL1 lysozyme, *E. coli* HB101 (pCIP54) cells harboring the mutated gene, *cpl1** (Fig. 1), expressed an active lysozyme when tested on pneumococcal cell walls (data not shown). The fact that the genetic manipulation did not alter the enzymatic activity of CPL1 lysozyme was a fundamental requirement for the successful construction of the novel chimeric enzymes.

Biochemical Characterization of the Chimeric Gene Products. Plasmids pCL and pLC contain the chimeric genes coding for a pair of reciprocal chimeric proteins between the pneumococcal amidase and the phage lysozyme (Fig. 2). Extracts obtained from *E. coli* HB101 (pCL) and *E. coli* HB101 (pLC) demonstrated the presence of lytic active enzymes (named as CLL and LCA respectively, hereafter) that degrade pneumococcal cell walls (Table 1). The CLL enzyme, which is built up by the N-terminal domain of the phage lysozyme and the C-terminal domain of the pneumococcal amidase, acts as a lysozyme and requires conversion to achieve full enzymatic activity—i.e., preincubation of the enzyme in the presence of choline or pneumococcal cell walls (15, 17, 18). On the contrary, the chimeric protein LCA was characterized as an amidase that did not require conversion to get maximal enzymatic activity (Table 1 and Fig. 2). To further characterize the chimeric gene products, we purified the enzymes to electrophoretical homogeneity following a single-step procedure previously used to purify the parental host and phage pneumococcal lytic enzymes, taking advantage of the affinity of these proteins for choline (18). It is

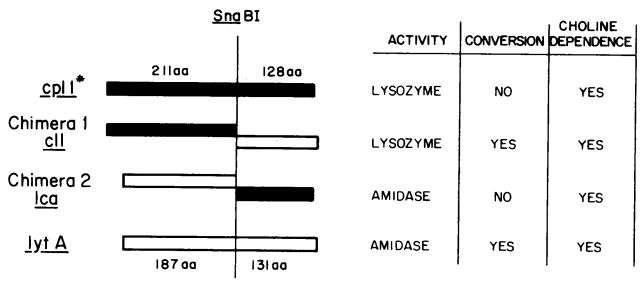

FIG. 2. Schematic representation of structures and some enzymatic characteristics of the pneumococcal amidase, phage lysozyme, and chimeric enzymes. The position of the restriction *Sna*BI site used for the construction of the chimeric genes is indicated as a reference. The numbers left or right of this restriction site indicate the amino acids extending to the N and C termini of the proteins.

Table 1. Activity and *in vitro* conversion by choline of the parental and chimeric pneumococcal cell wall lytic enzymes

	Enzymatic activity, cpm/10 min			
Preincubation	LYTA	CPL1	LCA	CLL
Without choline	342	2507	3728	240
With choline	3459	2699	3121	2067

Sonicated crude extracts were prepared from *E. coli* HB101 recombinant strains carrying the plasmids pGL80 (*lytA*), pCIP50 (*cpl1*), pLC (*lca*), or pCL (*cll*), respectively. These extracts were preincubated for 10 min at 4°C in the absence or presence of 140 mM choline. The samples were diluted in the assay buffer to avoid the inhibitory effect of choline, and their activities were assayed as described (15). The use of crude extracts is required to avoid the conversion of the enzymes by choline during the purification process.

noteworthy that the purified chimeric enzymes showed a thermal stability identical to that found for the parental enzymes (data not shown), suggesting that the newly created domainal interactions do not introduce significant alterations in the protein structure. The apparent M_r of the chimeric LCA enzyme was 36,000 (Fig. 3), which is in good agreement with the predicted value of 36,100. However, the chimeric CLL enzyme with an anticipated M_r of 39,300, slightly higher than that of the CPL1 lysozyme (39,000), had a mobility corresponding to a M_r of 38,500 (Fig. 3). This minor discrepancy could be due to an aberrant mobility of this enzyme in SDS/PAGE as already described for other proteins.

Like the parental enzymes, both the CLL and LCA chimeric proteins show an absolute requirement for the presence of choline in the cell wall substrate for activity. Replacement of choline by its analog ethanolamine in the teichoic acid completely abolished the lytic activity of the chimeric proteins (Table 2). The specific activities of the purified chimeric enzymes are in a comparable range to those of the parental lytic enzymes. The recruitment of the C-terminal domain from the CPL1 lysozyme by the amidase gave rise to an 8-fold increase in the K_m of the chimeric LCA amidase with respect to the pneumococcal amidase. On the contrary, the incorporation of the C-terminal domain of the amidase to the CPL1 lysozyme brought about a noticeable decrease in the K_m of the chimeric CLL lysozyme with respect to the parental lysozyme. On the other hand, choline, a noncompetitive inhibitor of the pneumococcal lytic enzymes (19), inhibited the activity of the CPL1 lysozyme at lower concentrations

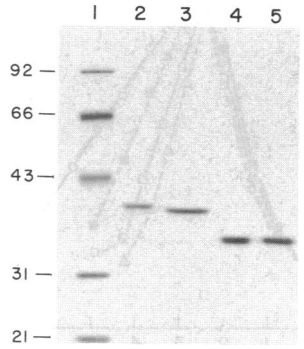

FIG. 3. SDS/PAGE analyses of the purified parental and chimeric pneumococcal lytic enzymes. The parental and chimeric enzymes were purified by a single-step procedure on DEAE-cellulose as described (18). The purified enzymes were electrophoresed on a SDS/10% polyacrylamide gel (29). Proteins were visualized with Coomassie blue. Lanes: 1, molecular weight markers (in kDa); 2, CPL1 lysozyme purified from extracts of *E. coli* HB101-(pCIP50); 3, CLL lysozyme purified from extracts of *E. coli* HB101-(pCL); 4, LCA amidase purified from extracts of *E. coli* HB101-(pLC); 5, pneumococcal amidase purified from extracts of *E. coli* RB791(pGL100).

Table 2. Biochemical properties of the purified parental and chimeric pneumococcal cell wall lytic enzymes

Lytic enzyme*	Property: Enzymatic activity Type‡	Enzymatic activity Specific§	Cell wall† Cho	Cell wall† Etn	K_m, g/liter	Cho IC_{50}, mM
LYTA	Amidase	600,000	+	−	0.04	24
CPL1	Lysozyme	750,000	+	−	0.09	3
LCA	Amidase	180,000	+	−	0.32	5
CLL	Lysozyme	800,000	+	−	0.03	20

*The lytic enzymes were purified as described in the legend to Fig. 3.

†Choline (Cho)- or ethanolamine (Etn)-containing cell walls were used as substrate for the enzymatic assay. −, Enzyme has <0.1% activity; +, 100% activity.

‡The type of enzymatic activity was determined as reported in *Materials and Methods*.

§Specific activity is expressed in units/mg of protein. Values shown are the means of three different experiments.

than those of the parental amidase (Table 2). This property was interchanged between the chimeric proteins (Table 2).

Biological Test. The purified chimeric enzymes were also tested for their capacity to lyse a culture of *S. pneumoniae* M31 strain, a mutant that has a complete deletion of the *lytA* gene, which makes this mutant unable to lyse at the end of the exponential phase of growth when incubated at 37°C (23). We have found that addition of CLL lysozyme or LCA amidase to the growth medium of M31 makes these cells lyse at the end of the exponential phase of growth (Fig. 4). This behavior mimics the spontaneous lysis found for the wild-type strain R6 at the end of the exponential phase of growth (11). These results demonstrate that (*i*) the chimeric lytic enzymes can be put under the same regulatory mechanisms of control operating on the parental lytic enzymes during the exponential phase of growth (11, 19), and (*ii*) they behave as efficient lytic proteins when tested *in vivo* in the homologous system. The fact that CLL lysozyme complements the *lytA* amidase defect of M31 strain parallels our previous finding that Cp-1 phage multiplied on M31 strain and the fact that CPL1 lysozyme was capable of successfully lysing this strain at the end of the exponential phase of growth upon addition to the medium (19). The complementation experiments strongly support the thought that enzymes with different catalytic activities (amidase or lysozyme) can play the same biological role—that is, the cell wall destruction—that results in the liberation of the phage progeny.

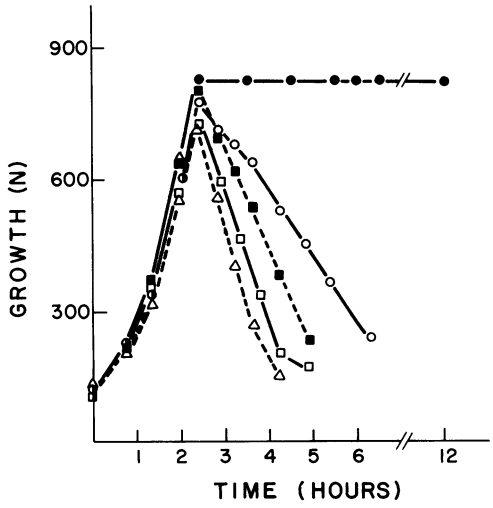

FIG. 4. Lytic effect of the chimeric enzymes on cultures of choline-grown *S. pneumoniae*. Cultures of *S. pneumoniae* M31 were grown in chemically defined medium (23) up to a cell concentration of 3.7×10^7 colony-forming units per ml. At zero time, the culture received 300 units of the purified CLL (△), LCA (■), pneumococcal amidase (○), or phage lysozyme (□) per ml, and the incubation was resumed. The growth of an untreated control culture of pneumococcus is also shown (●). Growth (or lysis) was followed by nephelometry (N).

DISCUSSION

The results reported here show interesting structural and functional relationships between the pneumococcal amidase and the CPL1 lysozyme. The formation of chimeric enzymes by genetic engineering manipulation of the *lytA* and *cpl1* genes demonstrates that (*i*) the active site of these enzymes lies within the N-terminal domain; (*ii*) the C-terminal domain is responsible for the recognition of the choline-containing cell wall substrate, for the peculiar activation of the pneumococcal amidase, and for the inhibitory effect of choline on these enzymes—functions only previously deduced from sequence homology (9); and (*iii*) the two domains are interchangeable and hence independently active. A result that reinforces the above conclusions is the finding that when the C-terminal domain of the pneumococcal amidase and the CPL1 lysozyme were cloned and expressed without any N-terminal domain, the truncated proteins conserved their affinity for choline (30). Interestingly, through the interchangeability of N- and C-terminal domains of the pneumococcal amidase and phage lysozyme, we create active enzymes, an exchange that has allowed us to effectively switch the regulatory properties of these enzymes without altering their catalytic activities. Although the changes observed in the primary structure of the C-terminal domain of the lytic enzymes (9) could account for such different regulatory properties, we cannot ascertain at the moment why this domain has divergently evolved in such a way that conversion only occurs in the pneumococcal amidase and not in the CPL1 lysozyme.

According to Houghton *et al.* (7), the chimeric enzymes suggest a number of evolutionary possibilities. These constructions provide an experimental model for the formation of ancestral genes by fusion of distinct genetic modules and a system for studying the molecular processes involved in cassette recruitment during evolution. In this sense, it has been pointed out that phages are products of modular evolution—i.e., the joint evolution of sets of functionally and genetically interchangeable elements each of which carries out a particular biological function (31, 32). This concept has also been found to apply to viruses of higher organisms (31, 33). Moreover, eucaryotic genomes seem to be constructed in a way that facilitates occasional rearrangement of DNA sequences to create new genes that code for protein domains in new combinations (34)—an organization that could serve to speed evolution by providing mechanisms for the generation of novel proteins from part of the old ones (35).

Despite the peculiar relatedness between bacteria and phages that could provide several examples of shared biological functions, very few cases of homology between bacterial and phage genes have been described (32). In the cases where homology has been found, it has been suggested that the bacterial and phage genes might be the result of a reciprocal interchange (36, 37). Although destruction of the bacterial cell wall by either bacterial or phage lytic enzymes has been considered as a classical example of a shared biological function, it was not until very recently that a case of homology between bacterial and phage lytic enzymes has been described (9). Based on the comparison of the sequences of the lytic enzymes of *S. pneumoniae* and its bacteriophages and on the hypothesis that changes in the cell wall structure could have provided the impetus for a type of "substrate-induced evolution" of lysozymes (38), we have

suggested that the peculiar presence of choline in the pneumococcal cell wall has acted as an element of selective pressure preserving the C-terminal modules of the amidase and CPL1 lysozyme that provide biological specificity to these enzymes (9). In fact, we have shown (9) that when the amino acid sequence of the lysozyme of the fungus *Chalaropsis*, an enzyme that degrades pneumococcal cell walls in a manner independent of the presence of choline, was compared to that of the CPL1 lysozyme, it was observed that only the amino termini of the two enzymes were similar. Furthermore, the recent finding that the lysozyme from the pneumococcal phage Cp-7, which is not regulated by choline, contains an N-terminal domain practically identical to that of the CPL1 lysozyme but a completely different C-terminal domain (39) also supports the idea that the pneumococcal lytic enzymes might be the result of an interchange of modules. Although domain swapping cannot be considered as a general cogent argument to prove that these enzymes could have evolved by the interchange of phage and bacteria modules, it demonstrates that these sequences fulfill the two basic properties of a module—i.e., interchangeability and functionality (31). Thus, it is conceivable that the laboratory model of domain swapping discussed here might have its counterpart in nature. It seems likely that CPL1 lysozyme could have evolved (*i*) from the fusion of a N-terminal module containing the catalytic domain derived from a pneumococcal phage genome, such as Cp-7, or (*ii*) from an ancestral lysozyme present in other microorganisms and a C-terminal module conferring the choline regulatory properties, which would have derived from the choline regulatory module of the pneumococcal amidase or other choline-dependent enzymes. The origin of the pneumococcal amidase might also be explained in a similar way. Nevertheless, we have not found so far an amidase capable of degrading pneumococcal cell walls that do not contain choline, which might represent the ancestral N-terminal module of the pneumococcal amidase. The acquisition of the C-terminal module providing the choline specificity to the pneumococcal cell wall-lytic enzymes seems to be a good example reinforcing the theory that molecular evolution might occur between genes by interchange of modules. The changes in substrate recognition and specificity should favor, under selective pressure, a better adaptation of the organisms to new environments. The establishment of experimental systems that allow the modular interchange of genes to create new proteins furnishes a basis to the extended thought that the modular organization might direct the evolution of most proteins (1, 2, 40). In this sense, our results could be best understood with the concept of bacteria as adaptable chimeras (41).

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1. Blake, C. C. F. (1978) *Nature (London)* **273,** 267.
2. Doolittle, R. F. (1985) *Trends Biochem. Sci.* **10,** 233–237.
3. Devlin, J. J., Devlin, P. E., Clark, R., O'Rourke, E. C., Levenson, C. & Mark, D. F. (1989) *BioTechnology* **7,** 286–292.
4. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1988) *Science* **240,** 1310–1316.
5. Nelles, L., Lijnen, R., Collen, D. & Holmes, W. E. (1987) *J. Biol. Chem.* **262,** 10855–10862.
6. Balganesh, T. S., Reiners, L., Lauster, R., Noyes-Weidner, M., Wilke, K. & Trautner, T. A. (1987) *EMBO J.* **6,** 3543–3549.
7. Houghton, J. E., O'Donovan, G. A. & Wild, J. R. (1989) *Nature (London)* **338,** 172–174.
8. Richards, J. H. (1986) *Nature (London)* **323,** 187.
9. García, E., García, J. L., García, P., Arrarás, A., Sánchez-Puelles, J. M. & López, R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 914–918.
10. García, P., García, J. L., García, E. & López, R. (1989) *Biochem. Biophys. Res. Commun.* **158,** 251–256.
11. Ronda, C., García, J. L., García, E., Sánchez-Puelles, J. M. & López, R. (1987) *Eur. J. Biochem.* **164,** 621–624.
12. Berry, A. M., Lock, R. A., Hansman, D. & Paton, J. C. (1989) *Infect. Immun.* **57,** 2324–2330.
13. Ronda, C., López, R., Tapia, A. & Tomasz, A. (1977) *J. Virol.* **21,** 366–374.
14. García, P., García, J. L., García, E. & López, R. (1986) *Gene* **43,** 265–272.
15. Tomasz, A. & Westphal, M. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 2627–2630.
16. Giudicelli, S. & Tomasz, A. (1984) *J. Bacteriol.* **158,** 1188–1190.
17. Briese, T. & Hakenbeck, R. (1985) *Eur. J. Biochem.* **146,** 417–427.
18. Sanz, J. M., López, R. & García, J. L. (1988) *FEBS Lett.* **232,** 308–312.
19. García, J. L., García, E., Arrarás, A., García, P., Ronda, C. & López, R. (1987) *J. Virol.* **61,** 2573–2580.
20. Boyer, H. & Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41,** 459–474.
21. Messing, J. (1983) *Methods Enzymol.* **101,** 20–78.
22. López, R., Ronda, C., García, P., Escarmis, C. & García, E. (1984) *Mol. Gen. Genet.* **197,** 67–74.
23. Sánchez-Puelles, J. M., Ronda, C., García, J. L., García, P., López, R. & García, E. (1986) *Eur. J. Biochem.* **158,** 289–293.
24. García, J. L., García, E. & López, R. (1987) *Arch. Microbiol.* **149,** 52–56.
25. Bolívar, F. (1978) *Gene* **4,** 121–136.
26. García, E., García, J. L., García, P., Sánchez-Puelles, J. M. & López, R. (1988) in *Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function*, eds., Actor, P., Daneo-Moore, L., Higgins, M. L., Salton, M. R. J. & Shockman, G. D. (Am. Soc. Microbiol., Washington, DC), pp. 218–223.
27. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7,** 1513–1523.
28. Sanger, F., Coulson, A. R., Barell, B. G., Smith, A. J. H. & Roe, B. A. (1980) *J. Mol. Biol.* **143,** 161–178.
29. Laemmli, U. K. (1979) *Nature (London)* **227,** 680–685.
30. Sánchez-Puelles, J. M., Sanz, J. M., García, J. L. & García, E. (1990) *Gene* **89,** 69–75.
31. Botstein, D. (1980) *Ann. N.Y. Acad. Sci.* **354,** 484–491.
32. Campbell, A. (1988) in *The Bacteriophages*, ed. Calendar, R. (Plenum, New York), Vol. 1, pp. 1–14.
33. Gibbs, A. (1987) *J. Cell Sci. Suppl.* **7,** 319–337.
34. Alberts, B., Bray, D., Lewis, J., Raff, M., Robers, K. & Watson, J. D. (1983) *Molecular Biology of the Cell* (Garland, New York).
35. Gilbert, W. (1978) *Nature (London)* **271,** 501.
36. Lauster, R., Trautner, T. A. & Noyer-Weidner, M. (1989) *J. Mol. Biol.* **206,** 305–312.
37. Stroynowski, I. T. (1981) *J. Bacteriol.* **148,** 91–100.
38. Jollès, P. & Jollès, J. (1984) *Mol. Cell. Biochem.* **63,** 165–189.
39. García, P., García, J. L., García, E., Sánchez-Puelles, J. M. & López, R. (1990) *Gene* **86,** 81–88.
40. Fox, S. W. (1984) in *Beyond Neodarwinism*, eds., Ho, M. & Sauders, P. T. (Academic, New York), pp. 15–60.
41. Sonea, S. (1988) *Nature (London)* **331,** 216.