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Isolation and Heteroduplex Mapping of a Lambda Transducing Bacteriophage Carrying the Structural Genes for Carbamoylphosphate Synthase: Regulation of Enzyme Synthesis in *Escherichia coli* K-12 Lysogens

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A N^- lambda bacteriophage transducing the structural genes for *Escherichia coli* K-12 carbamoylphosphate synthase (glutamine) (CPSase; EC 2.7.2.9) has been isolated and analyzed both genetically and physically. The whole *int-N* region is substituted for a short chromosomal segment corresponding almost exactly to the *car* locus. The study of CPSase, ornithine carbamoyltransferase, and aspartate carbamoyltransferase regulation in carriers of λ*dcar* confirms the previously reported participation of the *argR* gene product in the control of CPSase synthesis and points to the existence of a regulatory molecule involved in the control of both CPSase and aspartate carbamoyltransferase synthesis. The general usefulness of using N^- lambda transducing bacteriophages for the recovery of large amounts of gene products is discussed.

The structure of *Escherichia coli* K-12 carbamoylphosphate synthase (glutamine) (CPSase; EC 2.7.2.9) is specified by two adjacent genes: *carA* and *carB* (new symbol for *pyrA*, see reference 7). The *carA* subunit binds glutamine—the nitrogen donor for carbamoylphosphate synthesis (Fig. 1)—and displays glutaminase activity. The *carB* subunit performs the other steps of the reaction and contains the binding site of the allosteric effectors of the enzyme (7, 15).

CPSase synthesis is subject to a unique mode of control which is obviously related to the involvement of carbamoylphosphate in two major pathways, those concerned with arginine and pyrimidine biosynthesis; arginine and a pyrimidine (possibly more than one; see accompanying paper [8]) cumulatively repress the formation of the enzyme. Investigations of this regulation are being conducted at the in vivo and in vitro levels (3) with the help of a λ*dcar* transducing phage recently isolated by our group. The purpose of this communication is to describe the preparation of λ*car*, its physical structure, and the behavior of λ*car* carriers, from which models can be inferred regarding the mechanism of regulation of CPSase synthesis.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains and bacteriophage stocks employed are listed in Table 1. Genetic symbols are those from Taylor and Trotter (14). For special phage nomenclature, see references 5 and 16.

Phage assays and propagation. The procedures used for propagation and assay of phage have been described previously (6). In transductions involving λ*car*, the recipient strain was the *carB8* deletion mutant Jef8 (8). Car^+ transductants were selected on minimal medium plates supplemented with glucose (0.5% final concentration), L-methionine (200 μg per ml), and DL-threonine (200 μg per ml) but devoid of arginine and uracil. Titration of plaque-forming phages was performed on strain QD5003.

Isolation of λ*dcar*. Use of the *carB8* deletion mutant Jef8 allows a stringent selection for the Car^+ phenotype. The λ*car* phage was therefore isolated following the principle of the "mixed lysate" procedure (11) as described in reference 6. An *att*λ deletion, Car^+ strain (RW592) was used as recipient for the random integration of λ199 (Table 1) at secondary sites on the chromosome. A mixed lysate was prepared from several thousands of these lysogens. Strain Jef8 was used as a recipient to detect λ*car* phages present in the lysate. Two colonies producing high-frequency transducing lysates on further induction was recovered from a total of 2×10^{10} cells infected with about 6×10^{11} phages. They were later shown by heteroduplex (HD) mapping to carry iden-

tical *car* phages; only one isolate will be dealt with in the following paragraphs.

HD formation and electron microscopy. The preparation of the phage, the HD procedure, and the standardization of the measurements have been described in detail (6). Only the formamide technique of Davis et al. (2) has been used. Length measurements are reported in kilobases (KB; 1 KB = 1,000 bases of single-stranded deoxyribonucleic acid (DNA) or 1,000 base pairs of double-stranded DNA).

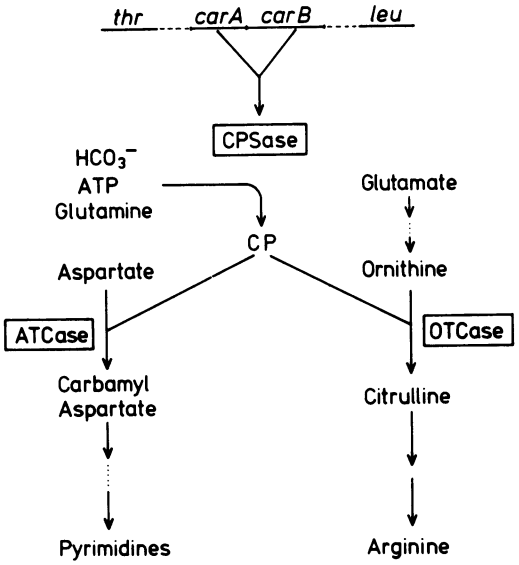

FIG. 1. *Involvement of carbamoylphosphate (CP) in arginine and pyrimidine biosynthesis in E. coli.*

Enzyme determinations. The assays for CPSase and ornithine carbamoyltransferase (OTCase: EC 2.1.3.3) have been described (9). Aspartate carbamoyltransferase (ATCase; EC 2.1.3.2) activity was determined as follows. The reaction mixture (0.6 ml final volume), containing tris(hydroxymethyl)-aminomethane acetate (pH 8; 50 mM), L-aspartate (20 mM), and [^{14}C]carbamoylphosphate (50 μCi/mM; 5 mM), was incubated for 20 min at 37 C. The reaction was stopped by the addition of 0.4 ml of 0.25 M trichloroacetic acid followed by 10 min of boiling to bring about decomposition of the remaining carbamoylphosphate into $^{14}CO_2$, NH_4^+, and inorganic orthophosphate. $^{14}CO_2$ was then eliminated from the liquid phase by 5 min of bubbling, and the precipitated proteins were removed by centrifugation. A 0.5-ml sample was added to 10 ml of scintillation fluid (9) and counted in a liquid spectrometer (Beckman model LS-100) with an efficiency of 55%.

RESULTS

Characterization of λ*dcar*. After centrifugation in a CsCl density gradient in the SW65 rotor, the transducing phage formed a band about 2 mm above the λ*199* helper and constituted nearly 50% of the particles present in the lysate. Transduction and assays of plaque-forming units performed with fractions sampled from such a gradient showed λ*car* to be defective on strain QD5003. The phage transduces both *carA* and *carB* strains (*rec*$^+$ or *recA*) to Car$^+$. In addition, it directs CPSase synthesis in vitro in a cell extract devoid of both *carA* and *carB* products (W. Lissens, N. Glansdorff, and A. Piérard, unpublished data). The phage thus

TABLE 1. *List of strains*

Strain	Relevant genotype	Source or reference
Bacteria		
RW592	*thi* Δ (*bio att*λ)[a]	R. Weisberg
QD5003	*supF*	R. Thomas laboratory
Jef8	*thr metB carB8*[b]	(7)
SA1550	*sup* lysogenic for λ*bio247 cI857 ΔH1*	C. Dambly, D. Court, and P. Brachet submitted for publication
Phages		
λ*199*	*cI857 susS7 sus xis6 Δb515 Δb519*	R. Weisberg
λ*nin5*	*N7 N53 C*$_{I}$*857 nin5*	(1)
λ*chi*	*chi126* (*chi* mutation is located in *cII* gene)	(4)
λ*chi*N	*N7 N53 red3 gam210 chi176*	Obtained by a cross between λ*NN red3 gam210* and λ*chi126*
λ*dcar chi126*		Obtained by a cross between λ*dcar* and λ*chi126*
λ*imm 434 sus*		R. Thomas laboratory

[a] Δ denotes deletion.

[b] The original Jef8 is (λ)$^+$. The strain used in most of the experiments reported here is a cured (λ)$^-$ derivative obtained by treating Jef8 (λ)$^+$ with the λ*hy imm21* phage.

carries the whole *carAB* region and is referred to hereafter as λ*dcarAB*.

The structure of λ*dcarAB* has been analyzed further by physical and genetic means.

HD mapping. HD molecules between λ*dcarAB* and the parent *199* show only one substitution loop (Fig. 2A). The exact location of the phage genes substituted by a segment of chromosome during the formation of λ*dcar* can be most conveniently determined from the structure of HD molecules formed between λ*dcar* and λ*nin5*. This combination indeed provides us with three easily recognizable markers: the *b515* and *b519* deletions present in the *b2* region of *199* – parent of λ*dcar* – but absent in λ*nin5*, and the *nin5* deletion, absent in λ*199* (Fig. 2B and 3A). The HD obtained (Fig. 2C and 3B) shows that the *b2* region of λ*dcar* is the same as in λ*199* and that the *nin5* region is intact. The results establish that the phage has substituted the *int-N* region for chromosomal genes. The coordinates indicate that the substitution affects the *N* gene itself, since the phage chromosome region situated to the right of the *car* genes represents only 25.4 ± 0.4% of wild-type λDNA, as computed from Fig. 2A and the length of the latter: 46.5 ± 0.5 KB (see reference 13 for a detailed physical map of the *attN* region where pL is estimated to be at 73.5% of the vegetative physical map).

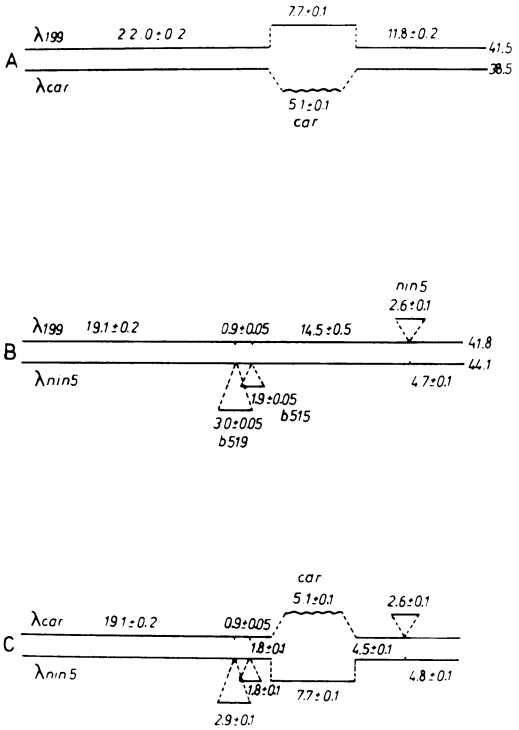

FIG. 2. *Schematic diagram of HDs studied. Coordinates are in KBs. Bacterial sequences are shown as a serrated line. Dashed lines connect points that are physically connected in the HDs.*

The length of the chromosomal segment carried by λ*dcarAB* is about 5.1 KB. Knowing the molecular weights of the two subunits of CPSase (172,000) and assuming an average molecular weight of 110 per amino acid, one can calculate that 4.7 KB could accommodate the two genes. There is thus very little more than *carA* and *carB* on the chromosomal segment carried by λ*dcar*.

Genetic characterization. λ*dcar* is completely unable to develop in any standard *E. coli* K-12 strain. Its presence is detected by its ability to yield Car$^+$ transductants with the *carB8* deletion mutant Jef8. To determine which phage genes are lacking from λ*dcarAB*, we performed complementation tests between a λ*dcar*$^+$ transductant and λ*imm434 sus* phages. As shown in Table 2, only λ*imm434 N*$^-$ phage burst remained as low in Jef8 (λ*dcar*)$^+$ as in Jef8. This result indicates that λ*dcar* does not synthesize the *N* product.

Other tests reinforce this conclusion. Indeed, like λ*N*$^-$, λ*dcar* does not establish immunity nor does it kill the host. Moreover, transductant strains segregate the Car$^+$ character at a high rate (after 20 generations, 50 to 80% of the culture is Car$^-$), indicating that the phage is in the state of a plasmid in the bacteria.

Further analysis has shown that the whole region to the left of *N* remains unexpressed in the λ*dcar* phage. This region (comprising notably genes *gam, red, del* . . .) being under *N* control, the tests were performed with a lysogenic strain that synthesizes the *N* product constitutively (SA1550, Table 1). A first observation was that λ*dcar* does not make plaques on this strain; like a λ*N*$^-$ *red*$^-$ *gam*$^-$ phage, it needs an additional *chi* mutation (4) to plate on SA1550. In addition, λ*dcar chi* grows perfectly well on the SA1550 lysogenic strain for P2 (Spi$^-$ phenotype [16]), confirming that *red* and *gam* genes are absent in λ*dcar* phage.

We conclude that λ*dcar* does not express any of the region extending from gene *int* to gene *N*, in full agreement with the heteroduplex data which show that this region is substituted by the *car* genes.

Regulation of CPSase, OTCase, and ATCase in λ*dcar* carriers. In lysogenic derivatives of the *carB8* deletion strain harboring both λ*dcar* and the helper, λ*199*, the regulation of CPSase appears to be normal when compared with a wild-type Car$^+$ K-12 strain (Table 3); the same is true for OTCase and ATCase. The control sites of the *carAB* cluster are thus carried

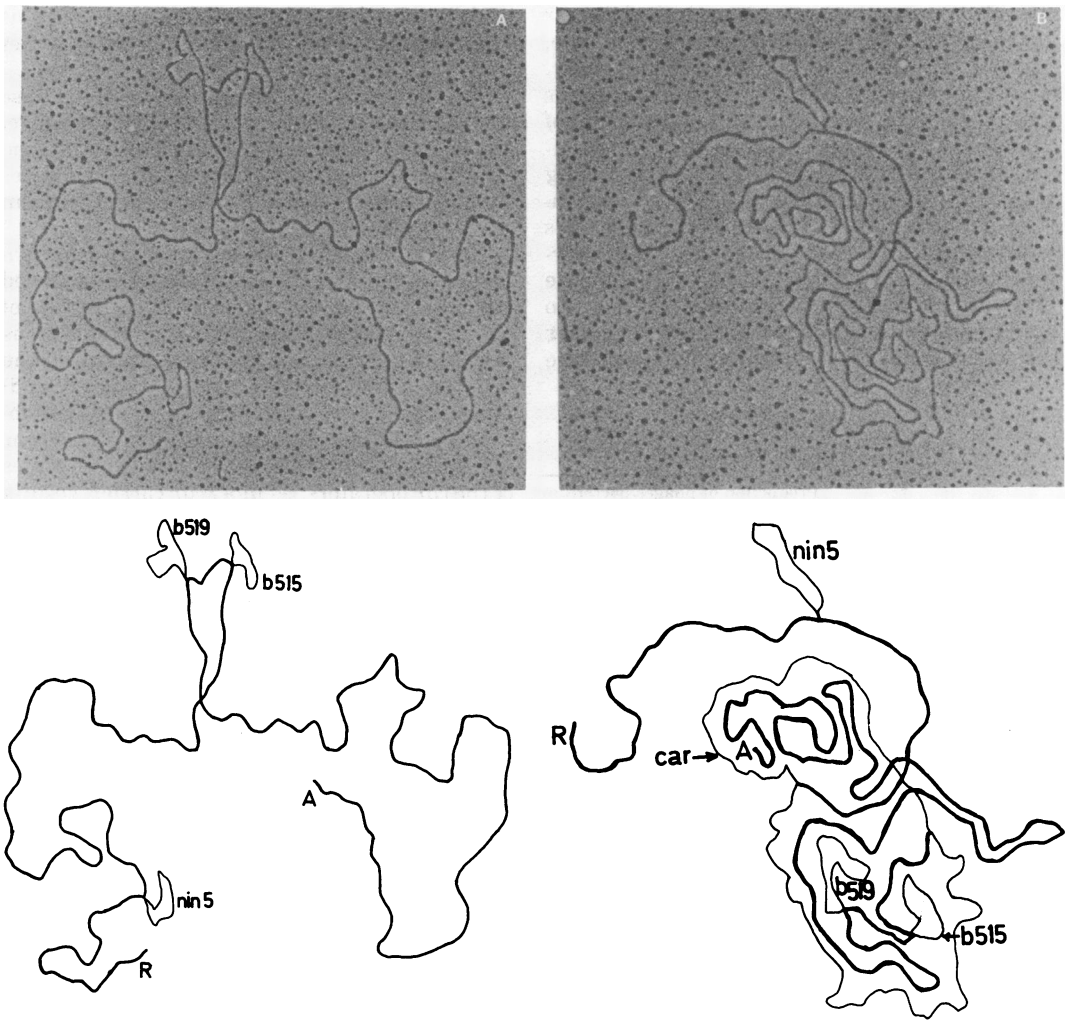

FIG. 3. (*A*) *HD between λ199 and λnin5*. (*B*) *HD between λdcar and λnin5*.

unaltered by λ*dcar*. When the *carB8* strain is infected by purified λ*dcar* in the absence of the helper, a very different picture obtains, summarized as follows (see data in Table 3).

(i) Considerable amounts of CPSase (10 to 25 times as much as in the wild-type strain) are produced in minimal medium; at the same time, a significant derepression of OTCase and ATCase is observed.

(ii) Cells maintained under conditions of repression by arginine and pyrimidines exhibit stable repressed levels of OTCase and ATCase; in contrast, a steady level of CPSase cannot be reached. This level keeps decreasing throughout the generations and eventually becomes zero.

(iii) As already noted above, the Car^+ character of λ*car* single carriers is unstable; the strain segregates Car^- cells after growth in the presence of arginine and uracil. This does not happen in the case of the (λ*car*)(λ*199*) double lysogen.

These properties are in fact expected from the genetic constitution of λ*dcar*. The phage is N^-, and of a non-leaky type, since all or part of *N* is deleted. A N^- phage is unable to express most of its functions but nevertheless displays a low, *N*-independent, expression of the replication genes *O* and *P*. The phage therefore multiplies as a plasmid in the cytoplasm (12). Up to 30 to 50 copies of a N^- plasmid have been reported to be present in any single cell of a carrier population (5).

If the number of λ*car* plasmids were high

enough to prevent repression control of the *car* genes by the amount of repressor available, "escape" synthesis of CPSase would occur. Since the repressor of arginine biosynthesis is known to play a role in the regulation of CPSase synthesis, either by itself or in combination with a regulatory molecule responding to certain pyrimidine derivatives (9), a concomitant derepression of OTCase is expected. It is interesting to note that in carrier cells overproducing CPSase, the synthesis of ATCase (first enzyme of pyrimidine biosynthesis) is also much higher than in Car$^+$ cells grown under the same conditions. Tryptophan synthetase and histidinol dehydrogenase were assayed as controls; they were not derepressed in either (λ*car*)(λ*199*) or (λ*car*) carriers.

The simultaneous derepression of CPSase and ATCase is, to the best of our knowledge, the first indication that CPSase regulation and the control of at least some of the *pyr* genes probably involve a common element other than the co-repressor itself.

Let us consider in more detail how such a molecular situation could result in the observed behavior. In minimal medium, only a part of the repressor molecules (of the pyrimidine as well as of the arginine pathway) present in the cells are in an activated (co-repressor-bound) state. These activated repressors are distributed between the *car* operators and the *arg* or *pyr* operators. Increasing the number of copies of the *car* operators, as in a λ*car* carrier, results in a decreased number of activated repressor molecules available for repressing the *arg* and the *pyr* genes: OTCase and ATCase are derepressed. After the addition of arginine and uracil, the number of activated arginine and pyrimidine repressor molecules will reach its maximum; at the same time, the plasmid will begin to segregate since it is no longer required for growth. How soon repressed rates of enzyme synthesis will become established depends on the actual increase in the population of active repressors after the addition of co-repressors, on the respective affinities of the repressors for their target operators, on the number of plasmids, and on the rate of plasmid segregation. For OTCase and ATCase, it seems that the repressed rate of synthesis is achieved shortly after addition of arginine and uracil; repression

TABLE 2. *Infection of Jef8 (λdcar) and Jef8 by λimm434 sus phages*[a]

Infecting phage[b]	Production of phages/bacterium after infection of:	
	Jef8 (λ*dcar*)	Jef8
λ*imm434 N⁻ N⁻*	0.2	0.2
λ*imm434 O⁻*	15	0.05
λ*imm434 P⁻*	24	0.6
λ*imm434 R⁻*	30	0.2
λ*imm434 F⁻*	25	0.1

[a] Recipient bacteria were infected by λ*imm434* phage at an infection multiplicity of 3, during the early-exponential growth phase in TB broth. After adsorption, the infected cultures were diluted 100-fold in TM broth and further aerated at 37 C for 90 min. At this time, $CHCl_3$ was added, and phage production was measured.

[b] λ*imm434 N⁻ N⁻* is more precisely λ*imm434 susN7 susN53;* λ*imm434 O⁻* is λ*imm434 sus08 red3 int;* λ*imm434 P⁻* is λ*imm434 susP3 red3 int6;* λ*imm434 R⁻* is λ*imm434 susR216 red⁻ int⁻;* λ*imm434 F⁻* is λ*imm434sus F204 red3 int6.*

TABLE 3. *Control of enzyme synthesis in λcar carriers*

Strain (relevant genotype)[a]	Addition to minimal medium[b]	Enzyme specific activities[c]		
		CPSase[d]	OTCase	ATCase
P4X (wild type)	None	21.8	1,270	232
	Arginine + uracil	0.7	51	34
P4XB2 *argR*	None	50.8	25,300	123
	Arginine + uracil	33.7	23,700	22
Jef8 *carB8* (λ*dcar N⁻*)$^+$(λ*199*)$^+$	None	19.5	1,480	304
	Arginine + uracil	1.7	68	38
Jef8 *carB8* (λ*dcarN⁻*)$^+$	None[e]	317.0	8,870	3,250
	None[e]	367.0	20,280	4,500
	Arginine + uracil[f]	38.0	52	23
	Arginine + uracil[f]	1.8	50	28

[a] Only the relevant genotype for this experiment is given here.

[b] Additions are made at the following concentrations (in μg/ml): arginine, 100; uracil, 50. All cultures were grown at 30 C.

[c] Specific activities are expressed as nanomoles of product formed per minute per milligram of protein.

[d] The glutamine-dependent activity of the enzyme has been estimated (see Materials and Methods).

[e] Two examples of cultures that have grown for many generations in minimal medium.

[f] Samples from independent cultures maintained in condition of repression by arginine and uracil for many generations.

of all copies of *car* genes present would probably become established during the same period; from then on, the rate of synthesis of CPSase should become proportional to the number of plasmids present in the cell. A way of evaluating the number of plasmids per cell is thus provided: a tentative estimation based on the comparison of the repressed differential rates of CPSase synthesis in the λ*car* carrier and in the wild-type strain suggests a number of 20 to 50 plasmids per cell. Nevertheless, the number of plasmids present in the cells of independent cultures is likely to vary somewhat; this would explain the variability of enzyme levels observed among cultures grown in minimal media, as well as in minimal media plus arginine and uracil.

DISCUSSION

We have isolated and described a defective transducing λ phage carrying a segment of the chromosome corresponding almost exactly to the *carAB* gene cluster. The isolation of those genes in the pure form will become possible as soon as another phage is prepared carrying on both sides of the *car* genes phage or chromosome segments different from those present on λ*dcar*; indeed, HD formation between such a phage and λ*dcarAB* would harbor the *car* cluster flanked by single-stranded insertion or substitution loops susceptible to cleavage by deoxyribonuclease SI (10). The present λ*car* has been successfully employed in the in vitro synthesis of CPSase (3) and for the detection of *car* messenger ribonucleic acid in a number of in vivo assays (unpublished data). New λ*car* phages are presently under study.

Being N^-, λ*car* is expected to behave as a plasmid in the cytoplasm of carrier cells. It appears reasonable to attribute the very high CPSase specific activities in λ*carAB* carriers to the multiplication of this plasmid and inadequacy of the amount of repressor available to control all *car* genes present. The simultaneous derepression of CPSase and OTCase supports this interpretation since the *argR* product is known to be involved in CPSase regulation (9). Moreover, the simultaneous derepression of CPSase and ATCase suggests that repression of *carAB*, *pyrB* and, presumably, other *pyr* genes involves the participation of a common element. It is therefore conceivable that the cumulative repression of CPSase synthesis is achieved by the cooperative action of two regulatory macromolecules: the above-mentioned element and the arginine repressor. They could act at adjacent sites on a bivalent *car* operator or participate in the formation of a compound molecule. The latter would have to bind both arginine and a pyrimidine to become fully activated.

Studying enzyme repression in cells carrying various *pyr* transducing phages would constitute an approach to unraveling the control mechanisms operating in pyrimidine biosynthesis.

N^- transducing phages behaving as plasmids would be useful for the purification of enzymes whose synthesis is controlled by repressors susceptible to binding by plasmid DNA or for the massive recovery of other gene products. Conditional *N* mutants would be particularly useful. They could be systematically isolated from cells carrying a deletion of *att*λ, a nonsense suppressor, and λ*Nsus* prophages inserted at secondary attachment sites of the bacterial chromosome.

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