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Genetic Control of Defective Cell Shape and Osmotic-Sensitivity in a Mutant of *Salmonella typhimurium*

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Summary. DA82, a complex strain of *Salmonella typhimurium* characterized by osmotic-sensitivity, spheric cell shape, and increased sensitivity to penicillin and cycloserine, was submitted to genetic analysis. Osmotic-resistant transductants that recover simultaneously normal cell shape and normal sensitivity to antibiotics were selected on low osmolality medium (NoS-A) at 43°. Complete revertants were not found; however, it was possible to isolate partial revertants displaying temporary arrest of cell division in NoS-broth, but no cell death. Those partial revertants retained round cell shape and sensitivity to antibiotics. Conjugation crosses located the mutation responsible for the lack of growth on NoS-A (*env-4*) in the region 102–108 min. No cotransduction with close markers was observed in tests mediated by phage KB1, yet, joint transduction of *env-4* with *argE, cod, aroC*, and *strA* was found employing phage P1. The gene order is *argE-cod-env-aroC-strA*. Thus, *env-4* appears to belong to the *Salmonella* locus homologous to *E. coli envB* locus. Isogenic strains carrying env^+ or *envB4* allele were prepared by transduction and studied. Mutation *envB4* produces abnormal cell shape, increases sensitivity to penicillin, cycloserine, deoxycholate, and UV irradiation; furthermore, it increases cell autolysis and modifies the phenotypic response to several carbohydrates on pH indicator media. *envB4* transductants show temporary inhibition of cell division in NoS-broth, but no exponential death. It is suggested that strain DA82 carries at least two envelope mutations: *envB4*, which is stable and pleiotropic, and an unidentified mutation which, in the presence of the *envB4* allele, causes exponential cell inactivation upon osmotic downshock.

Introduction

It has been observed in gram-negative bacteria that cell envelope defective mutants frequently display alterations in other properties besides that involved in their selection (Nagel de Zwaig and Luria, 1967; Egan and Russell, 1973; Wijsman and Pafort, 1974; Miyoshi and Yamagata, 1976). Pleiotropism of envelope mutations is not surprising when taking into account the complexity of the envelope and the biosynthetic and structural connections that have been found to exist among its different components (Costerton, Ingram, and Cheng, 1974). Yet, powerful mutagens are employed, in many cases, for the isolation of such mutants, therefore, the presumptive pleiotropic character of an envelope mutation should be always verified by genetic analysis.

A strain (DA82) displaying alterations in cell shape, osmotic behavior, and response to penicillin and cycloserine has been described in *Salmonella typhimurium* (Antón, 1972). The genetic analysis of DA82 was carried out in order to find out whether the abnormalities shown by the strain were all due to a single pleiotropic mutation or were produced by independent mutations.

This paper reports the location and characteristics of a mutation that modifies cell morphology; increases autolysis and sensitivity to antibiotics, deoxycholate, and UV irradiation; and changes the response to several carbohydrates. Results are also presented indicating that exponential cell inactivation after osmotic downshock, as shown by the osmotic-sensitive strain DA82, is caused by a second mutation which is dependent on the first for expression.

Materials and Methods

Bacterial Strains and Bacteriophages. All the bacterial strains are derivatives of *S. typhimurium* LT2. Their origin and properties appear in Table 1. Phage used for transduction KB1 (Boro and Brenchley, 1971) and phage P1CM (Silhavy et al., 1972).

Media. Complete medium was nutrient broth (Difco) + 5 g NaCl per liter (NB). Osmotic sensitivity was tested on broth with no NaCl added (NoS-broth). TY-broth: Bacto-Peptone Difco, 10 g; Yeast Extract 5 g; NaCl 5 g; distilled water 1 liter. Minimal medium was the E medium

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Table 1. Bacterial strains

Strain	Relevant markers	Source or derivation
LT2	wild type	P.E. Hartman
AroB	*aroB39 ara-9*	P.E. Hartman
AroC	*aroC32 ara-9*	P.E. Hartman
JL885	*cod-101 pyrC7 argE strA*[a]	J.L. Ingraham (Beck et al., 1972)
SA532	*cysE396 met-483*/F *cysE*$^+$	K.E. Sanderson
DA82	*envB4 osmA1 hisW1824 hisO1242 strA* (P22)	Antón, 1972
DA173	*envB4 osmA1 hisW1824 hisOG203 strA* (P22)	*hisOG203* phage × DA82, selection on min med. plus histidine at 43° (Fink et al., 1976)
DA183	*envB4 osmA1 leu-1256 hisOG203 strA*	NG mutagenesis of DA173
DA185	*envB4 osmA1 ser-1154 hisOG203 strA*	NG mutagenesis of DA173
DA203	*envB4 cys-1860 hisOG203 mal-591 strA*	M.R. Ferrari
DA216	*envB4 osmA*$^+$ *leu-1256 hisOG203 strA*	This paper
DA222	*envB4 cys-1860 mal-591 strA* F$^+$	SA532 × DA203
DA223	*envB4 mal-591 ara-9* F$^+$	DA222 × AroB
DA230	*envB*$^+$ *osmA1 leu-1256 hisOG203 strA*	This paper
DA245	*pro-852 envB4 mal-591 ara-9* F$^+$	DES mutagenesis of DA223
DA291	*metC104 envB4 hisOG203 strA* F$^-$	DA315 × DA185
DA294	*metC104 envB4 hisOG203 strA*	Treatment of DA291 with AO
DA315	*metC104* F$^+$	This laboratory collection
DA340	*cod-101 pyrC7 metC104* F$^+$	DA291 × JL885
DA355	*cod-101 pyrC7 argE strA*[a]	LT2 phage × JL885
DA357	*envB4 cod-101 pyrC7* F$^+$	DA245 × DA355
DA388	*argE pyrC7* F$^+$	DA245 × DA355
DA389	*argE pyrC7 strA* F$^+$	DA245 × DA355
DA395	*argE pyrC7 strA*	Treatment of DA389 with AO
DA396	*ser-1153 envB4 cod-101 pyrC7* F$^+$	DES mutagenesis of DA357
DA398	*his-6878 argE pyrC7 strA*	DES mutagenesis of DA395
DA411	*envB4 pyrC7 leu-1256 strA*	DA340 × DA413
DA413	*ser-1155 envB4 hisOG203 leu-1256*	Spontaneous in DA183
DA420	*envB*$^-$ *cod-101 pyrC7 metC104*	JL885 phage × DA413
DA424	*envB4 cod-101 pyrC7 metC104*	DA340 × DA413
DA467	*gal-1924 envB4 cod-101 pyrC7*	Spontaneous in DA357
DA468	*gal-1923 cod-101 pyrC7*	Spontaneous in DA420
DA475	*gal-1921 aroC32 ara-9*	Spontaneous in AroC
DA476	*galE1922 aroC32 ara-9*	Spontaneous in AroC
DA479	*argE pyrC7 metC104 gal-1923*	DA389-P1CM phage × DA468[b]
DA481	*envB*$^-$ *gal-1921*	DA411-P1CM phage × DA475[b]
DA482	*envB*$^-$ *strA gal-1921*	DA411-P1CM phage × DA475[b]
DA483	*envB4 gal-1921*	DA411-P1CM phage × DA475[b]
DA484	*envB4 strA gal-1921*	DA411-P1CM phage × DA475[b]
DA485	*envB*$^-$ *galE1922*	DA411-P1CM phage × DA476[b]
DA486	*envB*$^-$ *strA galE1922*	DA411-P1CM phage × DA476[b]
DA487	*envB4 galE1922*	DA411-P1CM phage × DA476[b]
DA488	*envB4 strA galE1922*	DA411-P1CM phage × DA476[b]
DA489	*envB4 cod-101 pyrC7 metC104*	DA467-P1CM phage × DA479

Only relevant markers are shown. For explanation on the symbols used see Sanderson (1972). In conjugation crosses the donor strain appears in the first place. Except for transductions with phage P1CM *clr-100*, which are specified, all other transductions were mediated by phage KB1. NG stands for nitrosoguanidine, DES for diethyl sulfate, and AO for acridine orange

[a] Strain JL885 appears to carry a mutation conferring salt dependence at 43° since it does not grow on NoS-A at 43°, but grows on the same medium at 37°, and on nutrient agar at 37° and at 43°. The locus involved is not the same as that present in DA82 because phage grown on the latter strain yields transductants on JL885 which grows on NoS-A at 43°. Moreover, strain JL885 shows normal cell shape. In order to avoid further complications in the mapping of *env-4*, strain DA355, a salt independent transductant from JL885, was employed in most cases

[b] Phage was obtained by thermal induction of a lysogenic derivative of the donor strain

(1956) with 0.5% dextrose. To test sugar utilization minimal medium M9 (Adams, 1959) with 0.5% of carbohydrate was employed. Solid media were obtained adding 15 g agar per liter to the corresponding liquid medium. Sugar utilization was assayed on plates of EMB Agar Base Difco containing 1% of carbohydrate, and, in some cases, on BCP medium containing Peptone Difco, 20 g; agar, 15g; Bromcresol purple, 0.025 g, carbohydrate 10g, per liter. Unless otherwise specified all the incubations on NoS-A plates were carried out at 43°.

Transduction and Conjugation Tests. Transductions with phage KB1 were carried out as described by Roth, Antón, and Hartman (1966) for phage P22. For isolation of P1 sensitive strains, preparation of P1CM *clr-100* lysates, and P1CM *clr-100* mediated transductions the procedures described by Mojica-A (1975) were followed. Conjugation tests were carried out as described by Antón (1968).

Osmotic Downshock. To test osmotic sensitivity the cells were grown to exponential phase in minimal medium. They were centrifuged, the pellet was rinsed with NB, and the cells were resuspended in a small volume of NB. Osmotic downshock was performed diluting samples of that suspension (0.1 ml) in prewarmed NB and NoS-broth (10 ml) so that cell density was about 0.8 1.0×10^7 cells/ml. The cultures, in 25 ml flasks, were incubated at 37° in a giratory water bath shaker, and samples were withdrawn at intervals and assayed for optical density (OD) at 650 nm and viable count. Plating for viability counts were carried out on TY-agar plates.

Sensitivity Tests. Sensitivity to penicillin (Penicillin-G, Squibb Argentina) and D-cycloserine (Mann Research Laboratories) was tested in TY-broth as described by Antón and Orce (1976). Sensitivity to deoxycholate (DOC) was scored on solid medium (TY-A plates) by the procedure described in that paper.

Autolysis in Buffer. Autolysis was assayed by the procedure described in Antón and Orce (1976). It is expressed as the decrease in OD (in percent) of cells maintained for 120 min in 0.05 M Tris-HCl, pH 8.5, at 37°.

UV Irradiation. Cells from either exponential or stationary cultures in TY-broth were centrifuged and resuspended in 0.1 molal NaCl to a final OD (650 nm) of 0.2. 5 ml of each suspension were placed in a 5 cm diameter petri dish, and UV irradiated with a General Electric Germicidal (G1578) lamp. Constant stirring of the cell suspension was maintained during irradiation. Survival was assayed on TY-A plates.

Results

Osmotic-Resistant Transductants from Strain DA82. Data already reported showed strain DA82 to carry several alterations. In addition to its round cell shape, DA82 was exponentially inactivated by transfer (osmotic downshock) from normal minimal medium (medium E) to low osmolality nutritive medium (NoS-broth), and displayed increased sensitivity to penicillin and cycloserine (Antón, 1972). It was also shown that the rate of osmotic inactivation increased with the temperature of incubation in NoS-broth. Furthermore, at 43° DA82 cultures lost the capacity for spontaneous recovery from osmotic inhibition shown at lower temperatures (Antón, 1972).

In order to facilitate mapping of the mutation controlling osmotic death, conditions for simple scoring of that characteristic were sought. It was found that strain DA82 was not able to grow on nutrient agar with no NaCl added (NoS-A) when incubation was carried out at 43°. It did grow at that temperature on nutrient agar containing salt (NA), or on NoS-A when the temperature of incubation was lowered to 37°. NoS-A allowed growth of wild type strains both at 37° and at 43°.

To test the capacity of NoS-A to act as selective medium for osmotic-resistant transductants, a transduction between strain DA183 (a *his leu* derivative of DA82) and phage KB1 grown on a wild type strain was performed directly on NoS-A plates. Taking into account a possible delay in the expression of the normal phenotype, some transduction plates were kept for increasing periods of time at 37° before being placed at 43° for two days. No colonies arised on the plates placed at 43° without preincubation at 37°, and few, if any, on the plates preincubated for one or two hours. However, numerous colonies appeared on the plates kept at 37° for three or four hours. Longer preincubations at 37° produced heavy background owing to growth of the recipient strain at that temperature. The colonies growing on NoS-A at 43° maintained the same nutritional requirements as the recipient, but showed normal cell morphology (Fig. 1 a, b) and normal response to 5 IU of penicillin/ml and to 20 μg of cycloserine/ml (Strain DA230, Table 5). Furthermore, they displayed normal osmotic behavior when transferred from E medium to NoS-broth (Strain DA230, Fig. 2).

Revertants of the Osmotic-Sensitive Strain. The appearance of revertants capable of growing on NoS-A at 43° was studied both in control cultures and after treatment with nitrosoguanidine (NG) and diethyl sulfate (DES). DA183 behaves as a very stable strain since in no case revertants showing normal growth on NoS-A were found. However, cultures treated with NG or DES produced a few tiny colonies on NoS-A. The cells in those small colonies retained DA183 round cell shape (Fig. 1c), and were sensitive to penicillin and cycloserine (Strain DA216, Table 5). However, even though transfer to NoS-broth produced a transitory inhibition of cell division in such "revertants", no significant cell death was observed (Strain DA216, Fig. 2), indicating that osmotic response was somewhat normalized.

The failure to find revertants with normal cell shape, osmotic behaviour, and antibiotic response could indicate either that a stable pleiotropic mutation was involved or that more than one mutation had to backmutate for DA82 to recover its normal

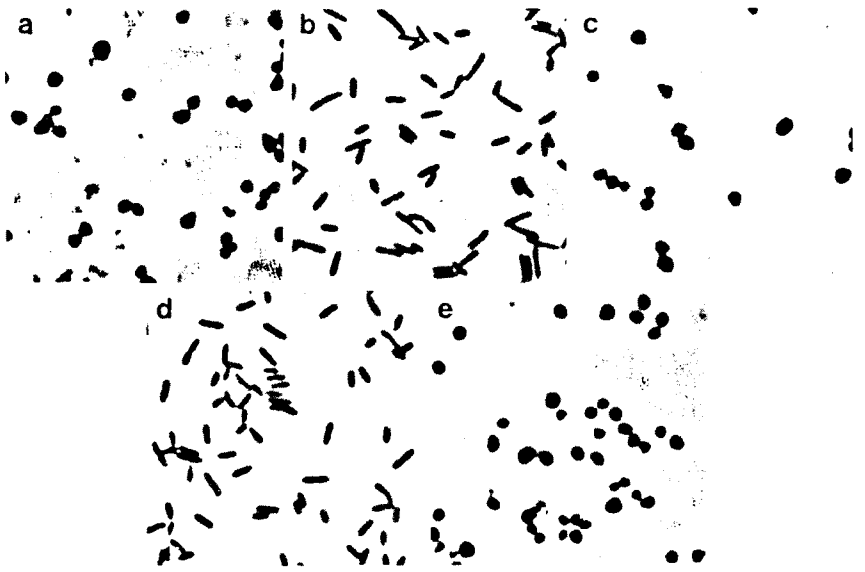

Fig. 1a–e. Phase-contrast photomicrographs of strain DA183, its derivatives, and isogenic *env-4* and *env*$^+$ transductants. The strains were grown on minimal medium plates supplemented with leucine and histidine, and the cells were resuspended in saline for observation. **a** DA183 (*env-4*); **b** DA230 (*env*$^+$ transductant from DA183); **c** DA216 (partial revertant of DA183); **d** DA482 (*env*$^+$ transductant from DA475); **e** DA484 (*env-4* transductant from DA475). Final magnification for all the micrographs, approximately × 1700

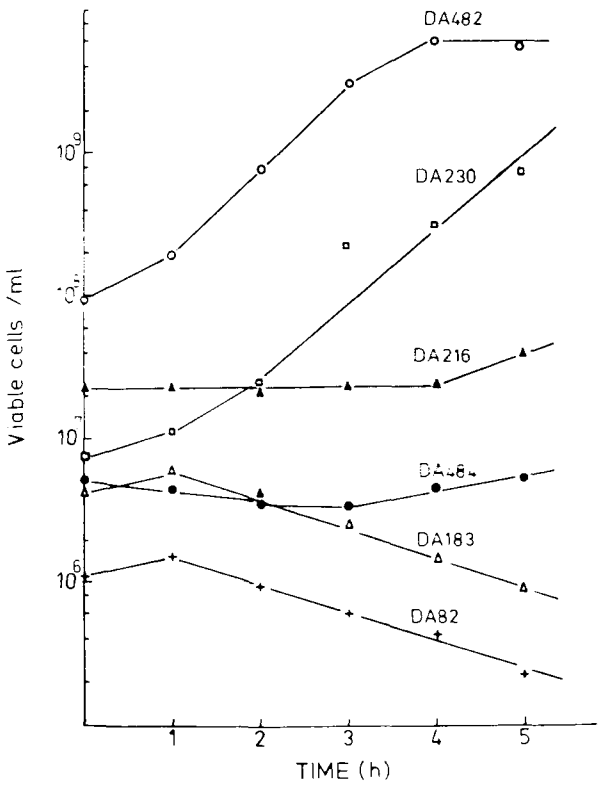

Fig. 2. Osmotic behavior of strain DA82 and related strains. The cells were grown in minimal medium, plus aminoacids when needed, and transferred, at time 0, to NoS-broth (see Materials and Methods). Symbols: (+) DA82 (*env-4*); (–) DA183 (*env-4*); (□) DA230 (*env*$^+$ transductant from DA183); (▲) DA216 (partial revertant of DA183); ( ) DA482 (*env*$^+$ transductant from DA475); (●) DA484 (*env-4* transductant from DA475)

phenotype. In this respect, the normal characteristics displayed by the transductants selected on NoS-A suggested that all the alterations were produced by the same mutation. Notwithstanding such results could be also explained assuming that there were two mutations, one of which produced abnormal cell shape and increased sensitivity to antibiotics, whereas the second mutation caused osmotic death, but only in those cells that also carried the first mutation. According to this assumption, the transductants selected on NoS-A received only the wild type allele for cell shape and antibiotic response, and retained, unexpressed, the osmotic-sensitivity mutation. The properties of the revertants isolated agree with the hypothesis outlined. The partial revertants would, thus, be backmutants of the osmotic-sensitivity mutation, and their failure to show complete osmotic normality would be due to the epistatic action of the stable cell shape mutation still present.

The above results and those reported below indicate that although the selective medium NoS-A was devised to conform the osmotic characteristics of DA82, it selects against round bacteria rather than against osmotic-sensitive cells.

For the sake of simplicity the mutation responsible for the failure of DA82 to grow on NoS-A will be hereafter called *env-4*.

Mapping of env-4. Preliminar mapping employing Hfr donors and auxotrophic DA82 derivatives as recipients demonstrated *env-4* to be located between

Table 2. Genetic analysis of recombinants from cross DA388 × DA294

Recombinant selected (No. of colonies)	Unselected markers			Frequency		No. of cross-overs required according to order		
	argE	*env*	*strA*	%	No. of colonies	1	2	3
metC$^+$ *pyrC*$^+$ (118)	+	−	+	47.5	56	2	2	2
	−	−	+	22.0	26	2	2	4
	−	+	−	18.6	22	2	2	2
	−	+	+	10.2	12	2	4	2
	−	−	−	0.8	1	4	2	4
	+	+	−	0.8	1	4	4	4
metC$^+$ *pyrC*$^+$ *strA* (114)	+	−		63.0	72	2	2	2
	+	−		21.9	25	2	2	4
	−	+		14.2	16	2	4	2
	+	+		0.9	1	4	4	2

Order 1: *metC---argE---env---strA*; 2: *metC---argE---strA---env*; 3: *metC---env---argE---strA*. Donor strain DA388 is *argE pyrC7* F$^+$; recipient strain DA294 carries mutations *metC104 env-4 hisOG203 strA*. All the recombinants were purified by single colony isolation before being tested for unselected markers

Table 3. Genetic analysis of *argE*$^+$ *serA*$^+$ recombinants from the cross DA396 × DA398.

Unselected markers			Frequency		No. of cross-overs required according to order	
cod	*env*	*strA*	%	No. of colonies	1	2
+	+	+	61.4	73	2	2
−	+	+	15.1	18	2	4
−	−	+	13.4	16	2	2
+	−	−	4.2	5	4	4
+	+	−	2.5	3	2	2
−	+	−	2.5	3	4	4
−	−	−	0.8	1	4	4
+	−	+	−	0	4	2

Order 1: *argE---cod---env---strA*; 2: *argE---env---cod---strA*. Donor strain DA396 is *ser-1153 env-4 cod-101 pyrC7* F$^+$; recipient strain DA398 carries mutations *argE pyrC7 his-6878 strA*. Total number of recombinants scored was 119

95 114 min according to the conventional linkage map of *S. typhimurium* (Sanderson, 1972). Further crosses employing and F$^+$ derivative of DA82 as donor and recipients carrying different auxotrophic markers in the region involved showed that *env* was located very close to *strA* (Ferrari, Figueroa, and Antón, unpublished results).

Transduction tests were carried out in order to check joint transduction of *env-4* with *strA* and several other neighboring markers. As DA82 and its derivatives carry prophage P22, phage KB1 was employed to perform the tests. When the phenotype Env$^+$ was selected on NoS-A plates, no cotransduction with markers *strA* (<0.1%), *aroB* (<0.5%), *aroC* (<0.44%), or *cod* (<0.2%) was found. The same result was observed when the reciprocal transductions were performed. No *env-4* colonies were found when *aroC*$^+$ (<0.5%), *cysG*$^+$ (<0.5%), *aroB*$^+$ (<0.5%), *cod*$^+$ (<0.1%), or *argE*$^+$ (<0.5%) were selected.

The failure to detect linkage by transduction prompted new attempts to obtain, by conjugation, a more precise location of *env-4*. To this purpose several strains carrying convenient markers were prepared and employed in further crosses. Table 2 shows the results from a cross between an *argE env*$^+$ *pyrC7* F$^-$ donor (DA388) and a *metC104 hisOG203 env-4 strA* recipient (DA294). Recombinants *metC*$^+$ *pyrC*$^+$ and *metC*$^+$ *pyrC*$^+$ *strA* were independently selected, purified by single colony isolation, and scored for unselected markers. Assuming that recombinant classes requiring two cross-overs should appear more often than those requiring four cross-overs, the frequency of recombinant types found suggested that the order of the markers involved was *argE-env-strA*.

In another cross between donor strain DA396 (*cod-101 pyrC7 env-4 ser-1154* F$^+$) and DA398 (*argE pyrC7 his6878 strA*) as recipient, scoring of unselected markers among recombinants *argE*$^+$ *serA*$^+$ indicated *env* to be located between *cod* and *strA* (Table 3). Thus, the gene sequence as shown by conjugation crosses would be *argE-cod-env-strA*.

As the distance between *argE* and *strA* is approximately 4 min (Sanderson, 1972), the lack of cotransduction between *env* and such close markers as *cod* and *strA* could be assigned to the small size of the KB1 transducing piece. Although no data on the length of KB1 DNA is available, it could be assumed to be similar to that of phage P22 because both phages give results alike in cotransduction tests.

Table 4. Frequency of joint transduction in crosses mediated by phage P1CM *clr-100*

Recipient	Donor	Selected marker	Unselected markers	No. of transductants analyzed	No. of transductants carrying unselected markers	Frequency of joint transduction (%)
DA475	DA411	*aroC*$^+$	*env*	195	18	9.2
DA476	DA411	*aroC*$^+$	*env*	250	34	13.6
DA475	DA411	*aroC*$^+$	*strA*	195	108	55.4
DA476	DA411	*aroC*$^+$	*strA*	250	144	57.6
DA475	DA411	*aroC*$^+$	*env strA*	195	2	1.0
DA476	DA411	*aroC*$^+$	*env strA*	250	6	2.4
DA476	DA488	*strA*	*env*	333	11	3.3
DA476	DA488	*strA*	*aroC*$^+$	333	190	57.0
DA476	DA488	*strA*	*env aroC*$^+$	333	11	3.3
DA476	DA488	*aroC*$^+$	*strA*	80	48	60.0
DA489	DA476	*cod*$^-$	*env*$^+$	118	23	19.5
DA468	DA411	*cod*$^+$	*env*	184	32	17.4
DA468	DA389	*cod*$^+$	*argE*	45	9	20.0
DA479	DA467	*argE*$^+$	*cod*	200	40	20.0
DA479	DA467	*argE*$^+$	*env*	200	2	1.0
DA479	DA467	*argE*$^+$	*metC*$^+$	200	0	< 0.5

All the transductants were purified by single colony isolation before being tested for unselected markers

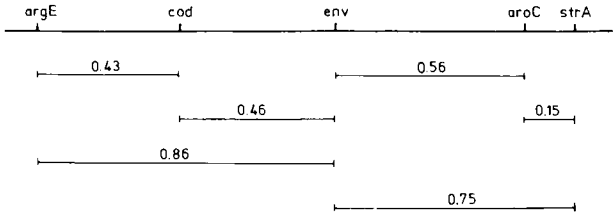

Fig. 3. Gene order and physical distances in the region *argE-strA*. Physical distances are expressed as fractions of the length of the DNA piece carried by a transducing particle of phage P1. They were calculated from the frequencies of joint transduction shown in Table 4, using Kemper's equation (Kemper, 1974)

It has been shown that some alterations in the lipopolysaccharide make *S. typhimurium* sensitive to *Escherichia coli* phage P1 (Okada and Watanabe, 1968a; Ornellas and Stocker, 1974). Phage P1 grows normally on those sensitive *Salmonella* strains and transduces genetic material between them in a fashion that resembles closely that observed in *E. coli* (Okada and Watanabe, 1968b; Enomoto and Stocker, 1974). As the DNA fragment carried by P1 is about twice the size of the piece carried by *Salmonella* phage P22, P1 has allowed detection of joint transduction between markers unlinked in P22-mediated transduction (Mojica-A, 1975). On this account, P1-sensitive derivatives of strains carrying *env-4* and other close located markers were isolated in order to check joint transduction in P1-mediated crosses.

Several transductions were performed with the results shown in Table 4. Joint transduction was observed between *env* and *cod*, *aroC*, *argE*, and *strA*. Furthermore, cotransduction was found between *argE* and *cod* which were not linked either in P22 (Beck et al., 1972) or KB1 mediated crosses.

The frequencies of joint transduction confirmed the gene order established in conjugation crosses, and allowed calculation of the physical distances between the genes by application of Kemper's equation (Kemper, 1974). The map obtained is depicted in Fig. 3. According to that data, as the length of the piece of DNA carried by P22 is about 40% of that contained in phage P1 (Mojica-A, 1975), genes *cod* and *argE*, and *cod* and *env*, would be just too distant to be jointly transduced by phage P22.

Properties of env-4 Strains. Out of the transductions between P1-sensitive derivatives of AroC (DA475 and DA476) and phage grown on an *env-4* strain, several *aroC*$^+$ transductants carrying either the mutant or the wild type allele of gene *env* were saved. Those isogenic strains, which probably differ only in gene *env*, should be free from all or most of the other mutations present in strain DA82. Therefore, they were employed to study the effects of the *env-4* mutation. Although the *gal* mutations isolated in AroC to make it sensitive to phage P1 were spontaneous, so that the strain was not mutagenized, transductants from two different *gal* derivatives were studied in order to compare their behavior. Furthermore, taking advantage of the proximity between *aroC* and *strA*, different combinations of *strA* and *env* alleles were also compared.

Cultures of *env-4 aroC*$^+$ and *env*$^+$ *aroC*$^+$ transduc-

Table 5. Properties of DA82, its derivatives, and some control strains

Strain	Growth on NoS-A	Cell shape	Growth on DOC (3 mg/ml)	Growth in		Autolysis (%)
				penicillin (5 IU/ml)	cycloserine (20 μg/ml)	
LT2	+	rod	+	+	+	19.8
DA82	−	round	−	−	−	38.9
DA183	−	round	−	−	−	37.4
DA230	+	rod	+	+	+	19.2
DA216	±	round	−	−	−	35.6
DA475	+	rod	+	+	+	23.0
DA476	+	rod	+	+	+	20.0
DA481	+	rod	+	+	+	23.7
DA482	+	rod	+	+	+	27.0
DA483	−	round	−	−	−	52.6
DA484	−	round	−	−	−	57.4
DA485	+	rod	+	+	+	22.7
DA486	+	rod	+	+	+	23.2
DA487	−	round	−	−	−	46.5
DA488	−	round	−	−	−	45.0

For details on the strains and experimental procedures, see text

tants in complete and minimal media were examined by phase contrast microscopy to check cell shape. It was found (Table 5) that all the transductants scored as *env-4* because of their lack of growth on NoS-A showed spheric cell shape whereas those displaying normal growth on NoS-A maintained their typical bacillar cell shape. *env-4* transductants retained their abnormal cell shape under all the nutritional and osmotic conditions tested, as DA82 did. Fig. 1 d and e show the cell morphology of a pair of *env*$^+$ (DA482) and *env-4* (DA484) isogenic transductants grown on minimal agar plates.

Sensitivity to penicillin and cycloserine was also tested. It was observed that *env-4* strains displayed sensitivity to penicillin (5 IU/ml) and to cycloserine (20 μg/ml) whereas, at the same concentrations, the isogenic *env*$^+$ strains were resistant to both antibiotics (Table 5). Table 5 also shows that all the strains with round cell shape, including DA82, DA183, and the partial revertant DA216, were sensitive to 3 mg deoxycholate (DOC) per ml. On the contrary, neither DA230 nor the *env*$^+$ isogenic strains were affected by that amount of DOC. Thus, it can be concluded that spherical cell shape and increased sensitivity to penicillin, cycloserine, and DOC are all pleiotropic aspects of *env-4*.

While screening for abnormal autolytic activity a group of envelope mutants isolated in this laboratory, it was observed that DA82, DA183, and DA216, all related *env-4* strains, displayed increased autolytic activity in alkaline buffer. When cells from those strains were placed in 0.05 M Tris-HCl buffer, pH 8.5, at 37°, there was a 40% decrease in OD after two hours of incubation. Wild type strains and strain DA230 showed, under the same conditions, a decrease of about 20% (Table 5). Such results suggested that increased autolysis was connected with the *env-4* mutation; therefore, the autolytic performance of the isogenic *env-4* and *env*$^+$ strains was tested. As can be seen in Table 5, *env-4* derivatives showed a very high autolytic level. On the contrary, and verifying the assumption that high autolytic level was due to the mutant *env* allele, *env*$^+$ derivatives retained the wild type amount of lysis.

Round cell mutants belonging to two different loci have been identified in *E. coli* (Matsuzawa et al., 1973; Westling-Häggström and Normark, 1975). Mutation in any of the two loci increases resistance to UV irradiation (Adler et al., 1968; Normark 1969; Matsuzawa et al., 1973). According to the mapping reported in this paper *env-4* stands in about the same place as the *envB* locus in *E. coli* (Westling-Häggström and Normark, 1975); therefore, response to UV irradiation was studied in two pairs of isogenic *env-4* and *env*$^+$ strains. Figure 4 shows the results obtained with cells of DA484 (*env-4*) and DA482 (*env*$^+$), grown in TY-broth. It can be seen that there was, in fact, a difference between the behavior of *env*$^+$ and *env-4* strains. The *env-4* mutation had, however, an opposite effect to that observed with *E. coli* mutants, since exponential cells of DA484 demonstrated to be more sensitive than those carrying the wild type allele. The same result was found when the isogenic pair of strains DA485 (*env*$^+$) and DA487

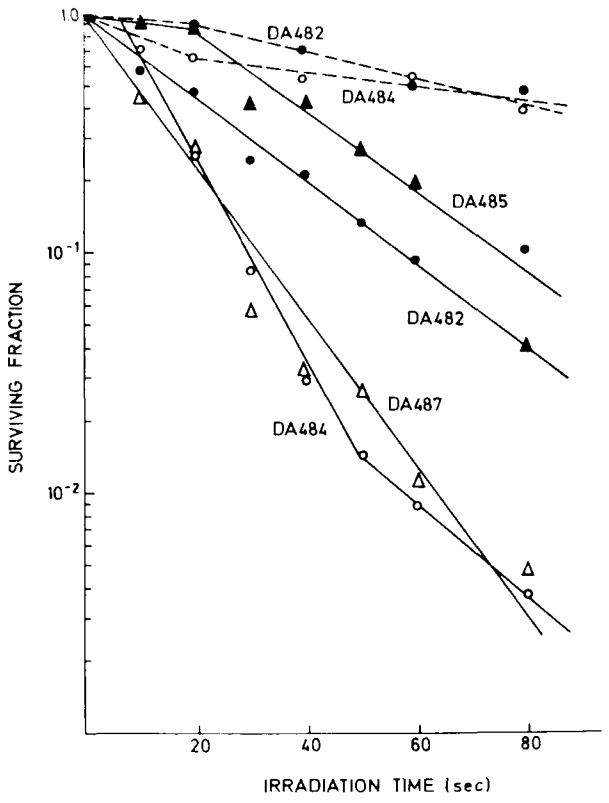

Fig. 4. Survival of isogenic *env-4* and *env*$^+$ strains after UV irradiation. Symbols: (○—○) exponential cells of DA484 (*env-4*); (●—●) exponential cells of DA482 (*env*$^+$); (△—△) exponential cells of DA487 (*env-4*); (▲—▲) exponential cells of DA485 (*env*$^+$); (○----○) stationary cells of DA484; (●----●) stationary cells of DA482

(*env-4*) was submitted to UV irradiation (Fig. 4). However, stationary *env-4* cultures (DA484) demonstrated about the same sensitivity as the corresponding *env*$^+$ cells (DA482) (Fig. 4).

One more characteristic affected by *env-4* was detected. Strain DA82 and all tested *env-4* transductants showed an anomalous phenotype on EMB agar containing either maltose, xylose, mannitol, mannose, or fructose. Instead of the typical greenish metalic sheen shown by DA230 and all the *env*$^+$ isogenic strains, *env-4* derivatives displayed an opaque dark red color. Red staining has been reported to develope by interaction between some cells having damaged envelopes and the eosin contained in EMB (Zinder, 1960). However, *env*$^+$ and *env-4* strains differed also when BCP medium, which does not contain eosin, was employed. Whereas *env*$^+$ strains turned bright yellow after 24 h of incubation on BCP plates, *env-4* strains required 48 h to adquire a pale yellow color. Preliminar tests have shown that *env-4* strain DA484 grows in minimal M9 medium with maltose or mannitol as sole carbon source.

Differences Between DA82 and the env-4 Transductants. The response to osmotic downshock of the strains obtained by transduction was investigated. As expected, *env*$^+$ strains showed no disturbance when transferred from E medium to NoS-broth (Strain DA482, Fig. 2). When the isogenic *env-4* strain DA484 was submitted to the same osmotic change there was a lag in cell division, with very little or no cell death, and after 3–4 h cell division resumed (Fig. 2). The same results were obtained with the isogenic pair DA485 (*env*$^+$) and DA487 (*env-4*). That is, although *env-4* strains constructed by P1-mediated transduction were temporarily affected by transfer to low osmolality medium, they did not suffer the exponential cell inactivation that characterizes the original *env-4* strain DA82 and its derivative DA183 (Fig. 2). In fact, the osmotic response of DA484 was similar to that observed with DA216, the partial revertant of DA183 (Fig. 2). These results agree with the hypothesis that the mutation responsible for osmotic cell death in DA82 is not *env-4* but another mutation which was not jointly transduced with *aroC*.

In addition to osmotic behavior, *env-4* strains differ from DA82 in the minimal amount of penicillin and DOC (cycloserine was not tested) that inhibits growth. Whereas *env-4* transductants DA483, DA484, DA487, and DA488, and the partial revertant DA216 were sensitive to 2 IU penicillin/ml and to 1 mg DOC/ml, higher concentrations (4 IU penicillin/ml, 1.5 mg DOC/ml) were required to inhibit growth of the original strain DA82 and its derivative DA183.

Discussion

Genetic analysis of strain DA82 has shown that at least two envelope mutations interact to produce the strain's phenotype. One of the mutations, named *env-4*, was mapped between markers *cod* and *aroC* by using *E. coli* phage P1. This phage allowed to detect cotransduction between *env-4* and genes *argE*, *cod*, *aroC*, and *strA*, which are too distant form *env-4* to be jointly transduced by *Salmonella* phage KB1. The effects of *env-4* were studied after transfer of the mutation, by transduction, to a new genotype. *env-4* has a very wide range of action. It produces spherical cell shape; increases sensitivity to penicillin, cycloserine, DOC, and UV irradiation; raises the level of autolysis; and causes phenotypic changes in the pattern of utilization of several carbohydrates.

env-4 is located at min 107, quite distant from min 78 where *divD*, the only other gene reported to cause round cells in *S. typhimurium*, has been mapped (Wyche et al., 1974). Furthermore, the properties of *env-4* strains differ sharply from those described for a *divD* mutant, which displays normal response to

penicillin, DOC, and UV irradiation (Wyche et al., 1974). On the other hand, the position of *env-4* corresponds to that found for *E. coli* gene *envB*. Gene *envB* has been located in *E. coli* between markers *aroE* (called *aroC* in *Salmonella*) and *aspB* (Westling-Häggström and Normark, 1975). Mutation in *envB* results in spherical cell shape; increased sensitivity to DOC, penicillin, and other antibiotics; and increased resistance to UV irradiation (Westling-Häggström and Normark, 1975). In spite of the opposite behaviour with respect to UV light, which could perhaps reflect allele or species peculiarities, the location of *env-4* and its properties suggest that this mutation represents the *Salmonella* locus homologous to *E. coli* gene *envB*. For these reasons, the mutation will be hereafter called *envB4*.

The presence in DA82 of a second mutation connected with osmotic inactivation is inferred from two observations. First, the isolation of revertants that retained abnormal cell shape but were not exponentially killed by transfer to low osmolality medium; and second, the properties of the strains prepared by transducing *envB4* to a new genotype. These strains, like the revertants just mentioned, were not inactivated by osmotic downshock although they displayed spheric cell shape. Hence, abnormal morphology was not enough to cause exponential death upon transfer to NoS-broth, therefore, another mutation had to be responsible for DA82 osmotic sensitivity. While *envB4* was stable so that no rod shaped revertants were in any case found, the second mutation, tentatively named *osmA1*, would backmutate producing DA216-like revertants. Furthermore, as the *envB*$^+$ transductants obtained from DA82, which should carry still *osmA1*, showed normal osmotic behavior as well as normal cell shape, it follows that *osmA1* is dependent on the presence of *envB4* for expression. The slighly lower sensitivity to penicillin and DOC shown by DA82 as compared to DA216 and the *envB4* strains prepared by transduction suggests that *osmA1* has pleiotropic effects too. At present, no direct information on the role played by *osmA1* in osmotic death is available.

It is not possible, at the present time, to visualize other relationship among the diverse properties affected by *envB4* than the structural and biosynthetic connections which are known to exist among the different components of the cell envelope. The effect of cell shape mutations on response to UV light appears to be quite different according to the species considered, the locus, and, probably, the allele involved. Two types of round cell mutants studied in *E. coli* K12 (*envB* and *rodA*) display increased resistance to UV (Normark, 1969; Matsuzawa et al., 1973). One of the loci (*divD*) identified in *S. typhimurium* does not modify response to UV (Wyche et al., 1974) whereas the one described in this paper, which presumedly corresponds to *envB* locus in *E. coli*, confers sensitivity to UV. It seems, therefore, that it is not the cell shape alteration but some other of the properties modified by such pleiotropic mutations which determines the effect on response to UV. The sensitivity of *envB4* strains to UV is not very high since survival decreases to only a tenth of that attained by *envB*$^+$ cells. Furthermore, it appears in exponential cells while stationary cells show about the same sensitivity as wild type bacteria. Perhaps the DNA of *envB4* cells is, at the replication point, more sensitive to UV or less amenable to repair than the DNA of *envB*$^+$ bacteria. As DNA is associated to the cell membrane at the replication point, this hypothesis could provide some explanation for the change in UV sensitivity observed in some envelope defective mutants.

envB is the second locus identified in *S. typhimurium* that increases autolysis in alkaline buffer. This property does not appear to interfere with normal growth of *envB4* strains, and is manifested only as a slight drop in OD and viability count in NB overnight cultures. Thus, *envB* mutants differ in this regard from *envD* mutants, which suffer a very significant autolysis in nutrient broth (Antón and Orce, 1976). The nature of the autolytic activity displayed by *envB* strains is not known. However, preliminar observations suggest that the same enzyme may be acting in *envB* and *envD* mutants. In fact, increased autolysis seems to be a rather frequent characteristic among envelope defective mutants (Antón, in preparation).

The significance of the phenotypic alterations observed in sugar utilization is not known. The changes are not due to interaction of *envB4* cells with the dyes in EMB (Zinder, 1960). Moreover, *envB4* strains are able to grow with maltose or mannitol as only carbon source. Actually, the aspect of *envB4* strains on pH indicator media suggests that carbohydrate utilization is perhaps retarded but not stopped. To explain the properties of *mem-1*, a membrane mutant of *S. typhimurium* which is unable to transport both phosphoenolpyruvate:glycose phosphotransferase system (PTS) sugars and non-PTS sugars (Cordaro, Postma, and Roseman, 1974), a general disturbance of sugar transport caused by the membrane mutation has been postulated (Cordaro, 1976). It remains to be investigated whether the behavior of *envB4* strains admits a similar explanation.

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