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ORIGINAL ARTICLE

Selection and evolution of mating products after spontaneous zygogenesis in *Escherichia coli* K-12

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Abstract The fate of products resulting from spontaneous zygogenesis (or Z-mating; complete genetic mixing in the absence of conjugative plasmid F) was investigated in crosses between *Escherichia coli* K12 strains carrying chromosomally inserted aminoglycoside resistance cassettes. The use of strains carrying genes conferring dominant resistance to drugs at the same site on the bacterial chromosome provided a new means to select diploid Z-mating products. It also allowed the existence of noncomplementing diploids exhibiting one parental or recombinant type to be confirmed. The formation and fate of Z-mating-derived complementing diploids could be investigated under conditions where loss of complementation is lethal, so as to eliminate colony growth due to intraclonal cross-feeding. Results show that the formation of complementing diploids could readily (i.e., within one to two generations) follow infection of either or both parents with an unidentified agent from an induced lysogenic *E. coli* strain 84SV carrying a presumptive Z-mating factor. The viable population in the mating mixture could be enriched in complementing diploids under some experimental conditions. A large series of subclonal analyses not only allowed the evolution of Z-mating products to be followed but also revealed unusual variations in drug-resistance marker expression in the descent of some doubly drug-resistant clones.

Keywords *Escherichia coli* · Genetic complementation · Phenotypic expression · Resistance to antibiotics · Unstable diploidy

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Introduction

Conjugation is a plasmid-mediated process involving unidirectional transfer of a segment of a copy of the chromosome (see Hayes 1970). However, the idea of cell fusion and heterodiploidization has been attractive since Lederberg (1949) and Zelle and Lederberg (1951) interpreted observations on genetic recombination in *Escherichia coli* as resulting from fusion between parental cells. Bacterial cell fusion can now be obtained by several artificial means (Schaeffer et al. 1976; Tyurin et al. 1997), and an unusual natural process generating complete diploids under conditions precluding classical conjugation was identified in *E. coli* K12. This unusual process, called spontaneous zygogenesis or Z-mating [originally called "illegitimate mating" (Gratia 1994)], is interpreted as a cell fusion-like process involving both plasmid pooling and cytoplasmic mixing (Gratia and Thiry 2003).

Physical evidence of Z-mating, seen as parental cells joined at their poles, is provided by immunofluorescence and electron microscopy after single and double labelling of DNA using immunocytochemical methods (Gratia and Thiry 2003). When the β-lactam mecillinam is used to block lateral elongation in a Z-mating mixture (so that each cell surface is reduced to a hemispherical pole), huge syncytium-like cells are formed that leave giant ghosts upon lysis (Gratia 2007a). "Spontaneous-zygogenesis-promoting" (Szp^+) strains are derived through Z-mating or through exposure of F^- strains to induced *E. coli* 84SV lysates, which presumably contains the genetic determinant of Z-mating. A preliminary analysis of the activity of crude 84SV lysates revealed that neither DNA, nor protease-sensitive proteins, nor plaque-forming phage particles could be implicated. It is suggested that conversion of F^- strains to Szp^+ status is conferred by an unidentified Z-factor transduced to F^- cells by a defective phage (Gratia and Thiry 2003). For such mating to occur, at least one parent must be Szp^+, but the other parent can be any non fertile F^-

E. coli strain or even a heterospecific partner (Gratia 2007b). The Szp^+ phenotype does not require any additional feature.

Diploids of *Bacillus subtilis* obtained artificially through polyethylene-glycol-induced protoplast fusion are most often noncomplementing (Hotchkiss and Gabor 1980; Grandjean et al. 1996, 1998). Although Z-mating products of *E. coli* are selected routinely for a complementing diploid phenotype, functional genetic complementation appears very unstable, and is followed by non-complementing diploidy (Gratia 1994, 2005, 2014). Based on the number of colonies formed on selective medium, the Z-mating frequency turns around 10^{-4}. Nevertheless, a frequency of 5–20 % has sometimes been obtained by counting sectored colonies (indicative of a phenotype switch) on nonselective medium (Gratia 2005).

Switching between noncomplementing forms was found to require a phase of complementation. Two events can be distinguished by the response to environmental stresses. The first consists of a final switch from one form to the other and is not influenced by incubation conditions. The other, revealed as a transient transfer of phenotypic expression from one genome to the other, can be triggered by heat shock or starvation (Gratia 2014).

The present work aims to investigate the formation and fate of Z-mating-derived complementing diploids under conditions where loss of complementation is lethal. This approach eliminates colony growth due to intraclonal cross-feeding. Competent Szp^+ cells are able to promote Z-mating after infection of partners of a cross with a transforming agent in lysates. The time required for their formation was assessed by submitting at intervals the mating mixtures to drugs for which resistance requires complementation. Subclonal analysis was then performed to follow the evolution of cells through the alternate expression of genomes in non-complementing diploids or serial recombination processes.

Materials and methods

Bacterial strains

The strains used are presented in Table 1. The Szp^+ strains MG405 and MG406 are derived from IL1 and IL4. The latter are F^- $LacY^-$ poly-auxotrophic AB1157 derivatives made resistant to kanamycin and gentamicin, respectively, by insertion of resistance cassettes. The cassettes carried aminoglycoside-modifying-enzymes genes joined to tandem copies of the *E. coli* Lac and Tn*10* Tet operators (*lacO* and *tetO*, respectively), to form *lacO* array *aphII* ($Kana^R$) and *tetO* array *aac2* ($Genta^R$) (Lau et al. 2003), into the same site of the chromosome (at 1801 kb, 200 kb from *dif*). The cassettes used show no sequence homology (C. Possoz, personal communication). The Lac^+ Trp^- gentamicin-resistant strain MG407 was obtained by Z-mating between MG406 and the KB Low strain KL262.

Media and chemicals

M9 buffer, broth (LB), nutrient agar (NA), minimal medium agar MA (M9 plus 1 mg glucose mL^{-1}), lactose-casein broth MLc (M9; 5 mg lactose mL^{-1}, 50 μg casamino acids mL^{-1}) and MLc-agar (here called LcA) were used. Thiamine, required by several strains because of their *thi-1* mutation, was added to all minimal media (1 μg mL^{-1}). M9-LB, containing 10 % LB, was used in crosses. When required, the liquid or solid medium was supplemented with 10 μg tryptophan mL^{-1}, 50 μg kanamycin sulphate (Km) mL^{-1} or 15 μg gentamicin sulphate (Gm) mL^{-1}, or with both drugs (KG). This aminoglycoside concentration was at least 2.5 times the minimum inhibitory concentration (MIC), as determined experimentally in LB at 37 °C.

Crosses and analysis of Z-mating products

As previously described (Gratia and Thiry 2003; Gratia 2005, 2007a, b, 2014), crosses were performed with at least one Szp^+ strain, and parental cultures were mixed at the optimal cell density, i.e., 10^6–10^7 CFU mL^{-1}. After a 30-min incubation in M9-LB, aliquots were plated on selective medium. Colonies of selected Z-mating products were counted after 72 h incubation. Conversion of F^- strains to the Szp^+ status was carried out by mixing 1 mL culture containing 1×10^8 CFU with 200 μL UV-induced lysate of *E. coli* SV84 for 20 min at 37 °C. Z-mating products were analyzed in highly diluted samples of bacteria picked up from selected colonies.

Experimental procedure for determination of survival

After a 30-min incubation of the Z-mating mixture, the culture was diluted twice for another 30-min incubation. LB medium was used for part of the experiments (where largely auxotrophic strains were used). MLc was used in cases where the parents differed in their nutritional requirements and lactose utilization so as to favor growth of complementing diploids. The culture was then split in two and 50 μg kanamycin mL^{-1} and 15 μg gentamicin mL^{-1} (KG) were added to one-half of each mixture, the other half serving as a control. After a 3-h incubation in the presence of the drugs (optimal for observing a differential effect), cultures were diluted (after centrifugation when necessary) and samples were plated on NA. The survival rate (S rate) was calculated by dividing the CFU count of each culture (Nt) by the initial CFU count (No)

Table 1 *Escherichia coli* strains used in this study

Strain	Pertinent genotype[a]	Conjugational phenotype	Source or derivation
W1S	*thr leu*	F^-	P. Fredericq
1485/Nx	*trpA,B his nalA*	F^-	Gratia 2005
GMS343	*lacY galK aroD argG rpsL mtl*	F^-	CGSC-5496 (G. Novel)
JC5519	*thr leu proA galK his argG recBC rpsL*	F^-	CGSC-5114 (A. Clark)
KL262	*galK pyrD trpC tyrA recA thyA*	F^-	CGSC-4322 (B. Low)
MG352	*lac galK rpsL metB*	Szp^+	Gratia 1994
MG355	*argG rpsL*	F^-	Gratia 1994
MG392	*trpA,B, nalA*	Szp^+	Gratia 2005
IL1	*thr leuB proA lacY galK hisG rpsL argE* [*lacO* array *aphII*]	F^-	Gratia 2014 (gift of C. Possoz)
IL4	*thr leuB proA lacY galK hisG rpsL argE* [tetO array *aac2*]	F^-	Gratia 2014 (gift of C. Possoz)
MG405	*thr leuB proA lacY galK hisG rpsL argE* [lacO array *aphII*]	Szp^+	Gratia 2014
MG406	*thr leuB proA lacY galK hisG rpsL argE* [tetO array *aac2*]	Szp^+	Gratia 2014
MG407	*galK trp pyrD tyrA* [tetO array *aac2*]	Szp^+	See Materials and methods
MG409	*trpA,B his nalA* [lacO array *aphII*]	F^- like	Gratia 2014

[a] Only mutations that are considered in the present experiments are indicated

of the minority parent in the control tube at the time of KG addition.

Results

The following sections describe the two methods of detecting the formation of Z-mating products.

Direct selection

Complementing diploids issued from a cross between auxotrophic strains marked with a resistance gene inserted at the same locus screened. Products of Z-mating between the Szp^+ Gm^R strain MG407 and the F^- Km^R strain IL1 were selected on LcA (for selection of lactose utilization by MG407 and biosynthetic abilities Trp^+ $PyrD^+$ by IL1). For double resistance selection, LcA was supplemented with both kanamycin and gentamicin (KG). The observed frequency of colony formation was close to 2×10^{-4} per minority parent on LcA alone and about 100 lower in the presence of KG. The differences between the results of nutritional and antibiotic selection highlight the instability of complementing diploidy. This suggests that growth of colonies selected on LcA was due mostly to intraclonal cross-feeding between non-complementing diploids and/or segregating haploids. When colonies formed on LcA were replica-plated on drug-supplemented medium, about 1 % were found to contain enough transient doubly resistant cells to elicit some visible growth.

Indirect evidence of complementing types

Table 2 shows the results of a typical S rate experiment, conducted to determine the effect of Z-mating on survival of bacteria exposed for 3 h to KG (i.e., following joint activity of genes inserted in cassettes with resistance markers). A non-mating mixture (M1) was obtained by mixing the F-strains

Table 2 Survival rate (S rate) of a Z-mating mixture between isogenic strains IL1 and IL4 and between their spontaneous-zygogenesis-promoting (Szp^+) derivatives MG405 and MG406 differing by their resistance to lethal drugs, either kanamycin (K) or gentamicin (G), when exposed to both

Set[a]	Total CFU mL^{-1} (Nt)[b]	S-rate Nt/No[c]	Ratio of S-rates M+ vs. M-
1. M1−	$1.3\pm0.5\times10^8$	3.1×10^1	
2. M1+	$1.0\pm0.4\times10^5$	2.4×10^{-2}	7.7×10^{-4}
3. M2−	$1.4\pm0.8\times10^8$	2.8×10^1	
4. M2+	$4.7\pm4.0\times10^6$	9.2×10^{-1}	3.3×10^{-2}

[a] A non-mating mixture of IL1 and IL4 (M1) and a Z-mating mixture of MG405 and MG406 (M2) were incubated in LB for 3 h with (M+) or without (M-) added KG (KG) before centrifugation and plating of samples (diluted or not) on NA

[b] Average of the different frequencies

[c] CFU mL^{-1} of the minority parent was 4.2×10^6 in M1 (IL1) and 5.1×10^6 in M2 (MG405)

IL1 and IL4, which differ only in the inserted drug resistance (i.e., Km^R and Gm^R, respectively). A Z-mating mixture (M2) was produced with the isogenic Szp^+ derivatives of the same strains, MG405 and MG406. In the presence of KG in LB medium, the S rate was nearly 40 times higher in M2, where Z-mating was expected, than in the non-mating mixture M1 (column 4, line 4 vs 2). Thus, Z-mating exerted a substantial protective effect.

An analogous experiment was conducted between strains carrying different nutritional markers, in addition to different inserted resistance cassettes. The culture with the mating mixture of MG407 and IL1 in M9-LB was diluted twice with MLc minimal medium (i.e., to favor diploids expressing the positive nutritional alleles of both parents). Complementing types (CT) CFU values resulting from functional genetic complementation for all tested markers (Table 3) were 43 times more frequent among survivors of the 3-h incubation with antibiotics than among untreated cells (last column: 6.8×10^{-3} vs 1.6×10^{-4}), even though the frequency of CFU with respect to the initial titer of the Szp^+ parent was only 1.6×10^{-5} in the treated mixture. This again reflects the massive selective killing of parental-type CFU, with relative protection of CT over a period equivalent to four to five generations of parental strains, despite the instability of complementing diploidy.

Minimal time required for functional genetic complementation between parental chromosomes after bacteria were infected with the Z factor vector

In the above sections, only one auxotrophic parent of the mating mixture was previously exposed to the 84SV lysate for conversion to Szp^+ status. The clone isolated as Szp^+ was then used for mating experiments and plating on selective medium. When F^- parental bacteria were mixed directly after exposure to the 84SV lysate, and plated on selective medium, colonies also appeared after a 72-h incubation. Successive events occurred during incubation: (1) generation of a Szp^+ bacterium after infection with the vector of the presumptive Z factor, (2) the Z-mating process itself, and (3) functional genetic complementation. On the basis of the results described above, the question of the timing of each event could be answered to some extent. A 3-h exposure to both drugs, requiring functional genetic complementation between the two arrays with *aac2* and *aphII* genes (for resistance to gentamicin and kanamycin respectively), will give rise to a significantly increased frequency of survivors at a given time after infection of one or both parent(s).

In these experiments, Km^R IL1 and Gm^R IL4 strains were exposed to the 84SV lysate in LB. After 20 min, treated bacteria were mixed for 30 min as usual, then the mating mixture was diluted twice in LB and incubated in several tubes for various times (from 1 to 3 h) before KG was added for 3 h. Controls were made with mixed non-infected IL1 and IL4 bacteria exposed to KG. The results depicted in Fig. 1 began to be significant in terms of higher survival when the infected cells were compared to non-infected cells already after 1 h, i.e., only one to two generations before KG was added to cultures. This means that formation of diploids with functional genetic complementation followed infection with the vector of the presumptive Z factor after a relatively short time.

Increase in CT frequency of the viable cell population of a Z-mating mixture

One might wonder to what extent segregation after zygogenesis masked the real frequency of CT. This question was partially answered by performing crossing experiments to test the effect of the drugs under conditions inhibiting growth and consequently segregation. When bacteria are maintained at 0 ° C for some time, during which there would be no division, the CTs previously selected for 3 h by KG persist longer than parental cell types (i.e., sensitive to Km or Gm), whether incubation on ice affected the killing activity of the antibiotics or not.

Two crosses, M_I and M_{II}, were then conducted. Z-mating mixtures, previously exposed or not to KG for 3 h, were

Table 3 Effect of drastic selection with lethal drugs on the frequency of complementing types (CT) in the viable cell population of a Z-mating mixture between strains IL1 and MG407 differing by resistance to drugs and by nutritional requirements

Exposure to KG	Colony count (Nt) after plating on[a]			Ratio Nt MG407-type /No[b]	Ratio Nt CT /No [b]	Frequency of CT CFU among survivors (NtCT/Nt MG407type)
	MA + P5 (IL1-type CFU)	LcA + UTT (MG407-type CFU)	LcA (CT CFU)			
−	$3.5\ (\pm0.8)\times10^{7}$	$2.6\ (\pm0.15)\times10^{7}$	$4.5\ (\pm2.0)\times10^{3}$	$2.7\ (\pm1.6)\times10^{1}$	$4.7\ (\pm2.0)\times10^{-4}$	$1.6\ (\pm0.5)\times10^{-4}$
+	$1.2\ (\pm0.6)\times10^{3}$	$2.2\ (\pm1.6)\times10^{4}$	$1.5\ (\pm0.9)\times10^{2}$	$2.3\ (\pm0.6)\times10^{-3}$	$1.6\ (\pm0.4)\times10^{-5}$	$6.8\ (\pm10.8)\times10^{-3}$

[a] The Szp^+ strain MG407, marked with *tetO* array *aac2*, was mixed with the F^- strain IL1, marked with *lacO* array *aphII* (respective average initial titres (No): 9.5×10^{6} and 1.6×10^{7} CFU mL^{-1}). The 30-min mixture of ca. 5×10^{7} bacteria in M9-LB was diluted twice in MLc for a 30-min incubation at 37 °C before addition (+) or not (−) of KG and an additional 3-h incubation. Plating was on the specified indicator media (P5: 200 μg hydrolysate of casein ("peptone 5") mL^{-1} for growth of IL1-like bacteria; UTT: uracil + tryptophan + tyrosine for MG407-like bacteria). Values are means of four experiments

[b] No. of MG407, the minority parent. Average of the different frequencies

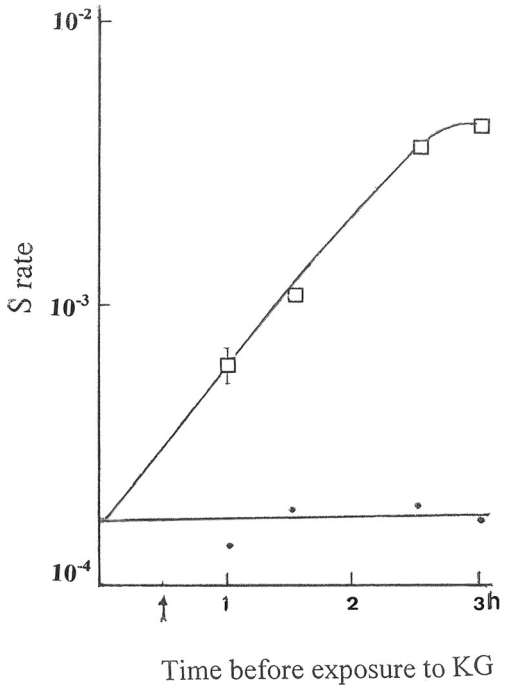

Fig. 1 Ability of induced 84SV lysates to promote Z mating. Bacteria of F-strains Km^RIL1 and Gm^RIL4 exposed to an induced 84SV lysate conferring the Z-mating factor were mixed in M9-LB (time 0). After a 30-min incubation in LB, the culture was diluted twice with LB and distributed in several tubes (*arrow*). A cocktail of KG was added at different times except to the control tube. The same was done with a culture of non-infected bacteria. The graph shows the values of the survival rate (S rate) to a 3-h exposure to KG, according to the time before treatment, in the set of infected (□) compared to not infected (●) mixtures

placed on ice for 25 h or more. The survival and frequency of mixed clones among survivors were compared. The CFU titre (Nt) of the drug-free cultures differed somewhat from the initial cfu titre (No), as it increased slightly for cultures from cross M_{II} after 25 h on ice, or decreased, i.e., it was two times lower for cultures from cross M_I after 43 h (Table 4, column 3, lines 1 and 3). However, survival in the drug-treated cultures M_I^+ was considerably lower (column 3, line 2). The same was observed for M_{II}. Whether the period on ice was shorter (25 h for M_{II}) or longer (43 h for M_I), a similar S rate ratio between treated and untreated cultures (ca. 2×10^{-5}) was observed (column 4). A series of about 100 well-separated colonies formed on NA by diluted samples of untreated cultures and by survivors of treated cultures were picked up, suspended in M9 and aliquots dropped on NA. After a 6-h incubation, bacteria were replica-plated on NA + Km and NA + Gm. In both cases, whether or not bacteria were exposed to KG, a portion of survivors was able to give rise to some growth on both media. However, the frequency differed notably, reaching 7×10^{-1} for bacteria exposed to KG, i.e., from 2.9 to 7 times that of non-exposed bacteria (column 5, lines 2, 4 vs lines 1, 3). Variance in the treated cultures was higher than in the cultures not previously exposed to KG (column 2), in agreement with the previous observation that Z-mating causes clumping and heterogeneity in terms of Z-mating products (Gratia 2007a). This is a supplementary argument in favor of the conclusion that diploids were frequent among survivors of treated cells.

Table 4 Effect of an incubation on ice on the survival of complementing diploids Km^R Gm^R [a] formed after a Z-mating between MG405 and MG406 (M_I) or MG406 and MG409 (M_{II})

Exposure to KG	Total CFU mL^{-1} (Nt)[b]	S rate (Nt/No[c])	Ratio of S rates M+ vs. M −	Frequency of mixed Km^R/Gm^R CFU among survivors
M_I-	$2.8\pm0.7\times10^6$	5.5×10^{-1}	2.8×10^{-5}	6.9×10^{-2}
M_I+	$7.9\pm3.8\times10^1$	1.55×10^{-5}		4.8×10^{-1}
$M_{II}-$	$4.6\pm1.4\times10^7$	1.8×10^1	1.55×10^{-5}	2.4×10^{-1}
$M_{II}+$	$7.2\pm5.9\times10^2$	2.8×10^{-4}		6.9×10^{-1}

[a] Cultures exposed to KG for 3 h were subsequently placed on ice for 25 h in the case of M_{II}, 43 h in M_I, before centrifugation and plating

[b] Mean of three tubes in M_I, four tubes in M_{II}

[c] No=5.1×10^6 CFU mL^{-1} of minority parent MG405 in M_I and 2.6×10^6 CFU ml^{-1} of MG406 in M_{II}

When colonies of mixed clones formed on NA were replica-plated on NA + KG, a large number of them grew and took on a patchy appearance. Yet, their analysis revealed that they were mixed and not doubly resistant recombinants. Their growth on NA + KG might have resulted from interactions between numerous cells of different resistance patterns in a very confined site. Therefore, drugs in cultures of low cell density massively eliminated parental types during the whole experiment, whereas the fraction of viable cell population existing as complementing diploids was enriched.

Analysis of Z-mating products derived from crosses between Km^R and Gm^R strains

An extensive subclonal analysis on purified products of Z-matings between various strains was performed. Observations included (1) segregation of both parental types (P1, P2) from CT clones isolated directly on MA, LcA or having survived the above-described enrichment step on ice in the presence of both drugs; (2) the appearance of recombinant types (R) among these subclones; and (3) the observation of phenotype switching (P1/P2 or P/R) between whole sets of markers. Analysis of 606 isolates corresponding to a first cycle of re-isolation of a total of 178 clones formed through eight different crosses, most of them having been repeated several times, showed some diversity in phenotypes for 6–12 genetic markers throughout the whole chromosome (Table 5). This diversity increased upon successive re-isolations.

The results of crosses 5–8 depicted in Table 5 involving resistance arrays were instructive and revealed some unexpected findings, as detailed below. After cross 8a between MG407 and IL1, isolates were backcrossed. Figure 2 concerns

Table 5 Subclonal analyses of various Z-matings products

	Cross between		Involved genetic markers[a]	Selected markers	No analyzed clones subclones[b]		No. of isolates of parental recombinant phenotype					Frequency of different phenotypes
	P1	P2										
	(Szp^+)	(F^-)					P1	P2	P1/P2	R_{1-n}	P/ R	
1	MG352	W1S	*thr leu lac galK rpsL metB*	$thr^+leu^+met^+$	5	25 (5)	4	2	5	12	2	0.20
2	MG352	JC5519	*thr leu proA lac his argG recBC rpsL metB*	$thr^+leu^+his^+$ arg^+met^+	4	14 (3)	5	5	4	0	0	0.20
3	MG352	MG355	*lac galK argG metB*	arg^+met^+	6	24 (4)	7	10	3	1	3	0.25
4	MG392	GMS343	*lacY galK trpA,B aroD nalA argG rpsL mtl*	trp^+aro^+	102	306 (3)	75	56	22	61	92	0.04
5	MG405	1485/Nx	*thr leu proA lacY trpA, B aphII nalA rpsL*	lac^+trpA,B^+	9	45 (5)	2	6	13	8	16	0.18
6	MG405	KL262	*thr leu proA lacY pyrD trpC his aphII tyrA thyA recA nalA argE*	lac^+trp^+ pyr^+thy^+	12	37 (3)	18	6	2	6	5	0.22
7	MG406	MG409	*thr leu proA lacY gal trpA, B aac2 nalA rpsL argE*	lac^+trpA,B^+	12	38 (3)	6	6	3	12	12	0.24
8a	MG407	IL1	*thr leu proA lacY trpC pyrD aroD aphII Aac2 his nalA rpsL argE mtl*	lac^+pyrD^+ trp^+aro^+	18	77 (4)	9	34	9	17	8	0.17
8b	MG407	MG405 (IL1 Szp^+)			10	40 (4)	12	5	8	8	7	0.27

[a] Order on the genetic map

[b] Mean number of isolates per clone in brackets

isolates from a third step mating mixture, which were able to grow on any complete medium containing either or both drugs. The parents, like those of the first step cross, differed in regard to their ability to ferment lactose. Thus it is possible to compare the expression of the lac^+ gene on the McConkey-lactose agar when this medium selected the expression of gentamicin resistance gene *aac2* linked to lac^+ in the MG407 chromosome and when it did not. The two plates to which doubly-resistant isolates were replicated looked completely different (i.e., one with Km and the other with KG). On the plate with Km only, lactose fermentation was not apparent (Fig. 2a), whereas the additional presence of Gm in the KG plate seemed to have 'induced' this biochemical process (Fig. 2b). Further analyses revealed that the latter observation resulted from a selective effect. Isolates expressed the *lac* mutation marking the chromosome with the kanamycin resistance gene *aphII*, but Gm exerted a selective pressure on Lac^+ bacteria, while part of the Lac^- cells were sensitive to Gm.

Figure 3, which concerns the same cross between strains MG407 and IL1, shows the various subclone types obtained after serial re-isolation cycles (e.g., the P2-to-P1 switch, extreme right). There was an unusually high frequency of subclones that recombined the resistance marker of one parent

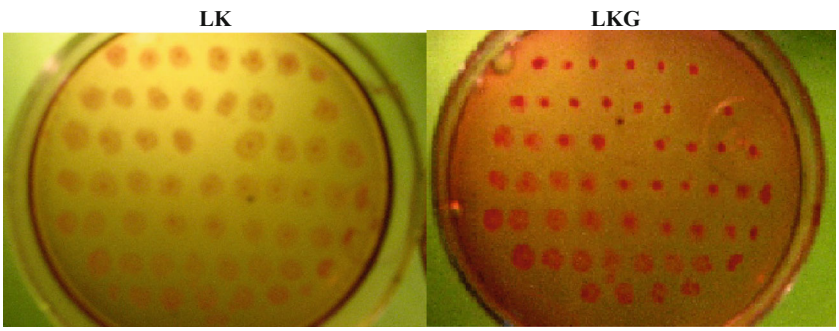

Fig. 2 Selective effect of gentamicin on a Z-mating mixture. Colonies of bacteria from a supernumerary Z-mating step after a cross between Lac^+ Gm^R MG407-like and Lac^- Km^R IL1-like bacteria and a 3-h exposure to kanamycin and gentamicin (KG). They were stabbed into NA and, after incubation, were replica-plated on two McConkey lactose agar plates, one containing 50 μg kanamycin mL^{-1} alone (LK) and the other 15 μg gentamicin mL^{-1} in addition (LKG). All colonies grew quite well as Lac^- on LK and more or less on LKG where some of the growing bacteria were Lac^+

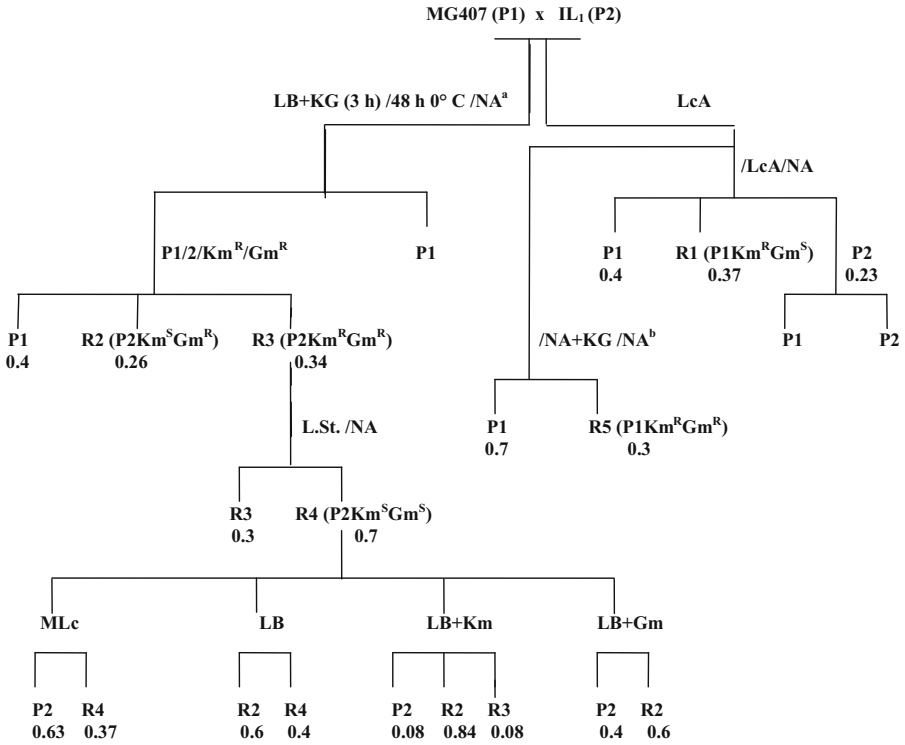

Fig. 3 The descent of Z-mating products (illustrative example). Illustrative results of the subclonal analysis of clones issuing from Z-mating between MG407 (P1) and IL1 (P2) and selected for biparental nutritional features (growth on LCA) or for transient double resistance to kanamycin and gentamicin. All isolates labelled P1 and P2 resembled parents Gm^R and Km^R, respectively. *R* Recombinant types, *R1* (*P1Km^{R}Gm^S*) recombinant phenotype characterized by kanamycin resistance (a marker of P2) plus all the nutritional (including fermentation) markers of P1; *R2* (*P2Km^{S}Gm^R*) recombinant phenotype characterized by gentamicin resistance (a marker of P1) plus all the nutritional markers of P2; *R3* (*P2Km^{R}Gm^R*) and *R5* (*P1Km^{R}Gm^R*) recombinant phenotypes characterized by all the nutritional markers of the indicated parent plus resistance to both kanamycin and gentamicin; *R4* (*P2Km^{S}Gm^S*) recombinant phenotype characterized by all the nutritional markers of P2 plus sensitivity to both kanamycin and gentamicin. *P1/2/Km^R/Gm^R* mixed subclone. *L.St./NA* Long stay on NA slopes (for several months at room temperature). [a]Mating mixture exposed to gentamicin and kanamycin for 3 h and kept on ice for 48 h. The colonies examined were isolated on NA and gave rise to positive replicas on NA + KG (see text). [b]Colonies on LcA replica-plated on NA + KG; re-isolation on NA of resistant bacteria

with all the nutritional markers of the other parent (R1 had recombined Km^R marking parent P2 with the nutritional markers of parent P1, whereas R2 exhibited the reverse), although recombination between other markers was sometimes observed (not shown). Any switching of the resistance marker in these recombinant types occurred "en bloc" with a whole set of associated markers, as expected for noncomplementing diploids. The appearance of doubly resistant subclones expressing all the markers of one parent plus the resistance marker of the other was surprising (R3 and R5). That is, homologous recombination between the resistance markers (inserted at the same site and displaying no sequence similarity) should not have occurred. Like similar subclones isolated from the rare colonies obtained after plating on LcA + KG (see above), these isolates proved stably resistant to both drugs, even when grown in drug-free medium.

Although the parents were stably resistant to either Km or Gm, some of these rare Km^RGm^R types became fully sensitive to any drug after a long stay on NA slopes (Fig. 3, R4: $P2Km^SGm^S$). Strikingly, this loss of resistance was only transient, since complete resistance to both drugs reappeared. This was observed while performing MIC tests. Tubes inoculated with less than 10^5 cells of the R4 isolate showed no growth after overnight incubation in the presence of either drug at twice the MIC of the sensitive parental strain. Growth could appear when the inoculum was greater and the incubation longer. This observation was considered further in the following experiment. The doubly sensitive R4 bacteria were incubated in minimal MLc broth, complete LB, and LB with added Km or Gm. Among the 65 subclones tested, 61 recovered resistance to one or both drugs, but this phenotype switch occurred without any concomitant change in the nutritional and fermentation markers of the different isolates. Also, the phenotype of 31 subclones did not correspond with the selection, as if there was no true selective pressure (Fig. 3, bottom), and neither was a plain connection between selection and phenotypes observed in isolates issued from other crosses.

Discussion

This work confirms that the frequency of observed Z-mating products depends on the medium used to select or visualize them. In crosses between IL1 and MG407, for instance, drastic selection for resistance to both kanamycin and gentamicin yielded a CT frequency approximately 100 times lower than that obtained with selection on the basis of the parental strains' combined nutritional abilities.

The frequency of diploid formation in Z-mating revealed by selection of nutritional abilities was highly variable (from 10^{-5} to 10^{-3} per Szp^{+} parent CFU) and below the frequency determined visually by counting sectored colonies (indicative of a phenotype switch), which may exceed 5 % (Gratia 2005, 2007b). It can be suggested that the formation of zygotes reflects the degree of activity of the putative Z-factor, which would be inactivated in a fluctuating fraction of the cell population.

The fact that Z-mating protects a substantial proportion of the cell population in an IL1 + MG407 mating mixture against the combined action of kanamycin and gentamicin, at least during a 3-h incubation in the presence of these drugs, confirms the assumption that the Z-mating frequency is higher than deduced from colony counts on selective media.

Previous experiments that consisted of respreading seeded plates at intervals have shown that expression of both parental genomes does not persist for long in the progeny of a complementing diploid (Gratia 2005). In fact, it seems that complementation seldom lasts long enough for a colony to form on a selective medium. In the case of Z-matings between parents with different auxotrophies, it has been suggested that the number of colonies selected for prototrophy and their variability in size reflect the number of generations in which the complementing state persists (Gratia 1994, 2005).

In the present work, which aimed to investigate the formation and fate of Z-mating-derived complementing diploids under conditions where loss of complementation is lethal, it was observed that far fewer colonies form when lethal drugs are used to select for complementation. This confirms the expectation that intraclonal cross-feeding between prototroph-derived non-prototrophic forms may contribute significantly to the growth of prototrophic colonies, whereas drug-sensitive forms emerging from doubly drug-resistant cells are killed.

The results presented here further confirm the existence of complementing types, since they were found to have combined double drug resistance with the nutritional markers of both parents. A population of such cells appeared to form rapidly and could be maintained for at least 3 h (i.e., four to five parental generations) under conditions expected to promote growth. Preferential killing of parental cell types during a further 25–43 h incubation on ice made it possible to obtain mixtures where complementing types represented at least two-thirds of the viable population.

In agreement with the observation of "fused-cell entities" observed by immunofluorescence or by electron microscopy (with single and double labelling of DNA) directly after a cross (Gratia and Thiry 2003), the use of cassettes marked by resistance markers allowed double labelling of DNA by another method, i.e., using cyan (CFP)/green (GFP) fluorescent protein-labelled repressors upon arabinose induction (C. Possoz and J-P. Gratia, unpublished).

The present experiments show that formation of diploids with functional genetic complementation followed infection with the presumptive Z factor after a relatively short time. The vector—possibly a defective phage—has to be identified through further analysis of the 84SV lysate. The Z factor gene (s) coding for membrane components (changes or addition of new ones) of the cell poles, which are concerned with Z-mating (Gratia and Thiry 2003; Gratia 2007a), could then be analyzed.

The pooling of pre-synthesized enzymes inactivating antibiotics in the cytoplasm of the presumptive zygote probably contributes to the rapid appearance of double drug resistance. Previous work (Gratia and Thiry 2003) has shown that lethal UV-irradiation of *recA* mutant cells can be rescued through Z-mating with rec^{+} cells. The timing and frequency of rescue suggest that the *recA* gene product is present from the moment the initial mating product forms, allowing prompt genetic recombination and DNA repair.

Among artificially produced *B. subtilis* exfusants, diploids are most often noncomplementing (Hotchkiss and Gabor 1980; Grandjean et al. 1996, 1998). Herein, the experiment depicted in Fig. 3 confirms by other means the existence of noncomplementing diploids in the descent of Z-mating products in *E. coli*. Moreover, in the present study, switching from the Km^{R} Gm^{S} to the Km^{S} Gm^{R} phenotype was associated with switching of whole sets of nutritional markers.

The appearance of $Km^{R}Gm^{R}$ clones expressing all the nutritional markers of only one parent (R3 and R5 in Fig. 3) remains unexplained. Indeed, additive recombination of the two arrays at the same site, when there is no homology between the arrays, should only be illegitimate and very infrequent. Some of these isolates additionally showed atypical variation in the drug resistance phenotype in the absence of any variation in other tested markers. Only stable $Km^{R}Gm^{R}$ isolates could possibly result from illegitimate recombination, in the case such an event might exist. On the other hand, isolates exhibiting variations in the resistance pattern could not result from such an event.

The observed phenotypic variations might result from transient silencing of resistance genes, independently from the expression of genes in other parts of the chromosome. These clones might therefore represent a new class of isolates issued from Z-mating products. For instance, it was observed that the complementing diploids exhibited a remarkable response to

the presence of lactose, when gentamicin was added in the medium (i.e., through expression of the chromosome carrying the *aac2* gene and the *lac*$^+$ allele). This could not have occurred, at least in such a way, using selection on minimal medium. Clearly, this highlights the need for more research on the mechanisms involved in inactivation and re-activation of chromosomes in diploids.

If the doubly resistant isolates are stably complementing meroploids, alternation between the two parental resistance characters could result from inactivation of either the parental or the recombinant chromosome. Yet, the double sensitivity observed in some isolates cannot be explained this way (i.e., inactivation of both chromosomes would result in non-viability). This raises the possibility of a third chromosome, deprived of both arrays through recombination and being the only one expressed in sensitive isolates. Reactivation of one or the other remaining silent chromosome could explain the resumption of resistance phenotypes (lower part of Fig. 3). Previous work has evidenced the re-activation of an "ancestral" silent chromosome through a succession of Z-matings, which suggests the occurrence of a noncomplementing triploid strain (Gratia 2005). The absence of selective pressure means that such re-activation did not depend on the presence of any drug.

All the observations on Z-mating raise the question of diploidy in bacteria in general, even when they are phenotypically stable haploids. Fusion of genetically heterologous cells leads to the gathering of two different chromosomes into the same entity, one being active and expressed and the other being inactivated. Reactivation of the latter then results in a phenotypic change. In the case of any ordinary strain with a stable phenotype, two identical chromosomes might coexist and undergo inactivation and reactivation of one chromosome. Still, such a switch would not be obvious unless a genetic modification occurs in one chromosome. The rough–smooth variation, for example, might be explained this way.

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