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Isolation and Mapping of *Escherichia coli* Mutations Conferring Resistance to Division Inhibition Protein DicB

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Temperature-sensitive *dicA* mutants of *Escherichia coli*, *dicA1*(Ts), are blocked for cell division, owing to derepressed expression of a division inhibition gene, *dicB*. We isolated mutants which survived a high temperature in the *dicA1* background and which survived induced expression of *dicB* carried by a high-copy-number plasmid. Most of the mutations conferred very slow growth on the cells. Two were mapped to the 90-min cluster of genes involved in translation and transcription, in or very close to gene *rpoB*. The majority of the other mutations were found to cause variable degrees of minicell formation and to map within or very close to the *minB* locus. Contrary to these mutations, the canonical *min-1* mutation did not confer resistance to DicB.

The *dicA1* mutation of *Escherichia coli*, which maps at 34.9 min on the standard genetic map (2), is responsible for a division-defective, temperature-sensitive phenotype (5). Previous studies have established that the *dicA* gene codes for a repressor (6). In the *dicA*$^+$ context, this repressor (which is homologous to the immunity repressor of bacteriophage P22) blocks the expression of two divergent promoters, *dicCp* and *dicBp*. *dicCp* is the promoter for a second repressor, DicC (homologous to the phage P22 Cro protein), while *dicBp* is the promoter for a complex operon (3, 4, 7). In the *dicA1* context, control by repressor DicA is substituted by control by repressor DicC which, under steady-state conditions, exerts little repression over *dicBp* (4). An analysis of the *dicB* operon established the presence of a gene (*dicB*) which, if placed under the control of the *lac* promoter, leads to inducer-dependent division inhibition. The sequence of gene *dicB* suggested the presence of two in-phase translation starts, coding for proteins of 12-kilodaltons ($DicB_L$) and 7 kilodaltons ($DicB_S$), respectively. Preliminary evidence based upon an in vitro coupled transcription-translation system supports the existence of both proteins (7). The sequence also indicated that *dicB* operon mRNA is likely to form a secondary structure preventing the expression of $DicB_L$, suggesting that $DicB_S$ could be sufficient to block division. Indeed, a plasmid that was used in the present study (pKC17) and that expresses $DicB_S$ alone under the control of the *lac* promoter was found to exhibit inducer-dependent division inhibition (7).

Gene-encoded division inhibitors have been found useful as probes for studying the genetic and biochemical controls of division. In *E. coli*, the interaction between the SOS-inducible division inhibitor SulA (22) and the product of the key division gene *ftsZ* has been established, mostly from genetic evidence (17, 20). In this paper, we report the isolation and mapping of mutations that confer resistance to DicB, as a step toward establishing its mechanism of action.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. Strains were grown in L broth supplemented with 20 μg of thymine per ml or in minimal Vogel-Bonner salts supplemented with thymine, 5 μg of thiamine per ml, 50 μg each of leucine and tryptophan per ml, and 0.5% glucose. Casamino Acids (0.2%) were added to this medium for transductions involving selection for tetracycline resistance. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml (50 μg/ml only when used in combination with 500 μg of methicillin per ml); tetracycline, 15 μg/ml; chloramphenicol, 20 μg/ml; and spectinomycin or rifampin, 100 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a concentration of 0.5 mM as an inducer of gene *dicB*.

Genetic methods. For the isolation of DicB-resistant mutants, strain JSP was grown in minimal medium at room temperature and treated with ethyl methanesulfonate (EMS) as described by Miller (21). The mutagenized culture was grown for 24 h at 24°C, and resistant mutants were isolated at 37°C on L-agar plates containing spectinomycin, ampicillin, and methicillin. For the physical mapping of mutations that confer resistance to DicB, a pool of approximately 1.5×10^4 Tn*10* insertions was inserted into strain CB0129 with phage λ NK561 (14) as described by Silhavy et al. (26). This pool was used to provide Tn*10*-linked markers by P1-mediated transduction. For the isolation of strain JS407, a spontaneous Ap^s derivative of JS324 (JS398) was isolated first. JS398 was cured of pAM1 by making use of the incompatible, temperature-sensitive, Cm^r plasmid pVF8 (kindly provided by V. François), and the resulting strain (JS406) was cured of pVF8 at 37°C to make strain JS407.

Other methods. Plasmid DNA was purified for analytical or preparative purposes as described by Ish-Horowicz and Burke (15). Cell lysates suitable for DNA restriction endonuclease analysis were prepared as described by Béjar and Bouché (5). For measurement of the percentage of polar divisions, strains were grown in L broth containing ampicillin and methicillin. IPTG, when present, was added at a final concentration of 0.5 mM. Septa were scored by direct inspection of successive fields containing 10 to 20 cells with a Leitz Ortholux II phase-contrast microscope at a magnification of 1,500×.

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid or phage	Relevant feature(s)	Origin or reference
Strains		
C600	*thi leu metA lac supE44 thr tonA21*	Our collection
CB0129	*thyA leu deoB* or *deoC thi supE44*	Our collection
Hfr B7	Hfr (PO 43) *metB relA*	Our collection
LN1390	CB0129 Δ*trpE63* (φ80 λ *imm ind*) *manA*	5
JS23	LN1390 *man*$^+$ *dicA1*	5
JSP	JS23(pAM1, pKC17)	This work
RS3032	*purB58 fadR613*::Tn*10 dadR1 trpA62 trpE6*	27
BW6165	Hfr P801 (PO 120) *argE*::Tn*10*	29
JS219	*dicA*$^+$ *malPp*::*lacI*^{q}	7
JS324	JSP *sbr-1*	EMS mutagenesis
JS325	JSP *sbr-2*	EMS mutagenesis
JS333	JS324 *sbr*$^+$ *malK*::Tn*10*	P1 transduction
JS334	JS324 *sbr*$^+$ *zij*::Tn*10*	P1 transduction
JS327	JSP *min-16*	EMS mutagenesis
JS328	JSP *min-20*	EMS mutagenesis
JS329	JSP *min-17*	EMS mutagenesis
JS330	JSP *min-18*	EMS mutagenesis
GC7237	*met hsdR gal supE sfiA85 sfiC*	16
GC7240	GC7237 *zcf117*::Tn*10 min-1*	16
GC7245	GC7237 *zcf117*::Tn*10 min-2*	16
GC7246	GC7237 *zcf117*::Tn*10 min-3*	16
GC7247	GC7237 *zcf117*::Tn*10 min-4*	16
MN42	HfrP4X *metB* Δ(*ppc-argECBH*)	12
JS412	MN42 *arg*$^+$ *sbr-1*	P1 transduction
JS398	JS324 Ap^s	Spontaneous derivation
JS406	JS398(pVF8) Cm^r Spc^s	pVF8 transformant
JS407	JS406 Cm^s	Curing at 37°C
Plasmids		
pGB2	*aadA*$^+$ (Spc^r) vector derived from pSC101	8
pAM1	*lacI*^{q} derivative of pGB2	D. Gil
pKC17	*dicB* 3′-terminal region in pUC9	7
pCL5	*malK*::Tn*10* derivative of pGB2	This work
pVF8	pSC101 derived, temperature sensitive, Cm^r	V. François
pNF1492	6.9-kilobase *Bgl*II fragment of λ d*rif*^{d}18 in pBR322	13
pNF1931	10-kilobase *Hin*dIII fragment of λ d*rif*^{d}18 in pBR322	13
pGR2017	Same as pNF1931 but *rpoB*$^+$ (Rif^s)	R. Glass
pAM1-N[a]	*rpoC*$^+$ Δ*rpoB* derivative of pNF1931	R. Glass
Bacteriophages		
λ NK561	*b*221 *c*I::Tn*10 O*am29 *P*am80	14
λ d*rif*^{d}18	*c*I857 *S*am7 d*rif*^{d}18	18

[a] The original designation of this plasmid is pAM1. It was named pAM1-N in this study solely to distinguish it from the *lacI*^{q} derivative of pGB2.

RESULTS

Isolation of DicB-resistant mutants. Our goal in isolating DicB-resistant mutants was to characterize the gene or gene product whose activity is blocked by the inhibitor. We hypothesized that the DicB target was likely to be an essential gene product and that resistant mutants would be infrequent. On the other hand, the frequency of spontaneous temperature-resistant (Ts$^+$) colonies appearing in the *dicA1* background is high (2×10^{-6} [5]). We presumed, therefore, that most of the spontaneous revertants would be defective in DicB synthesis rather than resistant to the inhibitor.

To ensure an efficient selection, we looked for mutations conferring resistance to DicB expressed simultaneously from the chromosomal copy in the *dicA1* background (Ts$^+$) and from the gene cloned episomally under the control of the *lac* promoter (IPTG^r). The strain used for this purpose, JSP (Table 1), carries, in addition to the *dicA1* mutation, a plasmid overproducing the *lac* repressor and a second plasmid coding for the C-terminal peptide (DicB$_s$) of gene *dicB* under *lac* control (7). Ts$^+$ IPTG^r mutants occurred at a frequency of less than 10^{-9} without mutagenesis, and although IPTG^r mutants were obtained at a frequency of 5×10^{-7}, they resulted from mutations in plasmid pKC17 in all instances tested. After EMS mutagenesis, Ts$^+$, IPTG^r, and double mutants were obtained at frequencies of 8.5×10^{-3}, 1.5×10^{-3}, and 6×10^{-5}, respectively.

Preliminary characterization of DicB-resistant mutants. A first group of approximately 50 resistant colonies was partially characterized by microscopic examination, and a few subclones were further characterized for their growth behavior, morphology in liquid cultures, and location of the mutations. On the basis of these results, a more systematic survey of 67 resistant colonies was made. The following classes could be distinguished. Forty-five colonies belonged to a class of very slowly growing strains (see below). All of these mutants, although isolated as IPTG^r, showed numerous filaments in the presence of 0.5 mM IPTG, indicating partial resistance only. In addition, 23 of these mutants showed defective division when grown at 37°C. Ten additional mutants had generation times comparable to those of

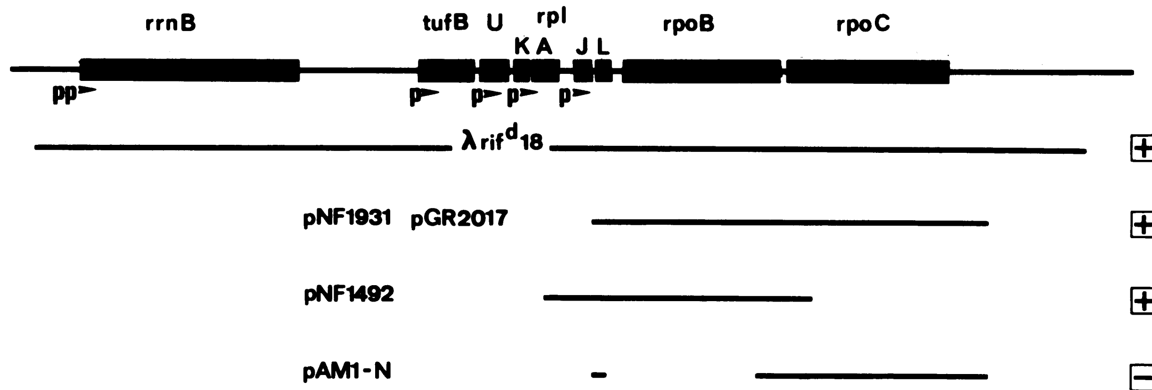

FIG. 1. Map of the 90-min region of the *E. coli* chromosome, indicating the positions of the major genes, promoters, and segments contained in the phages and plasmids discussed in the text (drawn to scale). The ability or inability to complement the *sbr* mutation is indicated by + and −, respectively.

the wild type but showed polar divisions associated with size heterogeneity, reminiscent of the phenotype of *min-1* mutants (1). One of these mutants formed minicells in the presence of IPTG only, and four other mutants were partially Ts. The remaining mutants (12 of 67) were distributed among different phenotypic classes and were not studied in any detail.

Genetic mapping of the locus responsible for slowly growing (*sbr*) DicB-resistant mutants. The genetic locus responsible for resistance to DicB and slow growth is referred to as *sbr*. A typical representative of this class, strain JS324 (*sbr-1*), had a generation time of 300 min in Luria broth at 26°C, as compared with 60 and 65 min for strains JSP and JS333 (parent and wild-type recombinant, respectively [Table 1]) grown under identical conditions. This difference was less pronounced at higher temperatures, since the plasmid-free, isogenic strains JS407 (related to JS324) and LN1390 (parent of JSP) had generation times of 68 and 28 min, respectively, at 37°C. To map the *sbr* locus, we transferred Tn*10* by P1-mediated transduction from a pool of random insertions (see Materials and Methods) into strains JS324 (*sbr-1*) and JS325 (*sbr-2*). These two strains show no residual filamentation at 37°C. Tc^r colonies were selected at room temperature. Approximately 1 colony of 300 colonies (13 of 3,700 for JS324 and 2 of 600 for JS325) appeared after 2 to 3 days; the remainder appeared after 1 week only. All fast-growing Tc^r colonies had become IPTG^s and showed extensive filamentation at 37°C. Two Tn*10* insertion strains, JS333 and JS334, obtained in JS324, were further studied. A phage P1 lysate made on strain JS333 (Table 1) showed that the Tn*10* insertion in this strain had a 10% cotransduction frequency with *sbr-1*. To locate Tn*10*, we took advantage of the fact that since *Bam*HI cuts within Tn*10* and leaves the *tet* locus intact, chromosomal DNA adjacent to Tn*10* may be cloned by selecting for Tc^r hybrid plasmids. DNA from JS333 was digested with *Bam*HI and ligated to *Bam*HI-treated DNA of vector pGB2 (8), and plasmids from Tc^r Spc^r-transformed colonies were analyzed. A restriction map for the 15.5 kilobases of chromosomal DNA carried by one of these plasmids, pCL5, was constructed and compared with the chromosomal map of Kohara et al. (19). Good agreement was found over more than 15 sites within the 4,320- to 4,335-kilobase interval of the map, and the data suggested that Tn*10* was inserted in *malK* at 91.5 min (24). In agreement with this hypothesis, JS333, contrary to JS324, was found to be Mal$^-$. The Tn*10* insertion in JS334 was too close to a *Bam*HI site to be mapped by the same method but showed quantitative cotransduction with sbr^+ in both JS324 (60 of 60) and JS325 (45 of 45), demonstrating that these mutations in these strains are allelic. Strain BW6165 carrying *argE*::Tn*10* (89.5 min) was also used to refine the location of *sbr*. Of 41 Tc^r transductants (from JS324), 40 had reverted to sbr^+. This result, together with the data obtained from the *malK*::Tn*10* insertion, suggested that *sbr* could map within the cluster of genes involved in translation and transcription at 89.8 to 90 min (2). This hypothesis was confirmed upon the isolation of Rif^r λ d*rif*^{d}18 (17) (Fig. 1) double lysogens in JS324. All these colonies appeared within the same time as sbr^+ colonies, and all of them (20 of 20) had regained sensitivity to IPTG or to incubation at 37°C.

To further refine the location of *sbr*, we tested several plasmids carrying all or part of the operon for core RNA polymerase for their ability to restore the Sbr$^+$ phenotype. For this purpose, a plasmid-free derivative of JS324, JS407, was first isolated (see Materials and Methods). JS407 was then transformed by plasmids pNF1492, pNF1931, pGR2017, and pAM1-N (Fig. 1) or plasmid pBR322, with selection for resistance to 100 μg of ampicillin per ml at 25°C. pNF1492 (Rif^r) contains genes *rplJ*, *rplL*, and *rpoB*. pNF1931 (Rif^r) and pGR2017 (Rif^s) contain genes *rplL*, *rpoB*, and *rpoC*. Because *rpoB* does not appear to have a specific promoter (see reference 23 for a recent discussion), *rplL* is presumably coexpressed with *rpoB* from the *tet* promoter of the pBR322 plasmid vector. pAM1-N is a deletion derivative of pNF1931 that contains a fusion of genes *rplL* and *rpoB*. This plasmid expresses *rpoC*, presumably from the *tet* promoter. Transformants appeared as colonies of uniform size after 48 h for pNF1492, pNF1931, and pGR2017 and had regained temperature sensitivity, indicating complementation of the mutation. Complementation was not observed in the cases of pAM1-N and pBR322. From these results, we conclude that the *sbr-1* mutation likely maps within the gene for the beta-subunit of RNA polymerase, although a location within *rplL*, which precedes *rpoB* and codes for ribosomal subunit L7/12, is not entirely ruled out.

Mutation *sbr-1* was moved from strain JS324 to strain MN42 by cotransduction with *argECBH*. Strain JS412 is one of a minority (7 of 37) of colonies which appeared after 3 days at 37°C on selective plates containing all amino acids except arginine. JS412 (the methionine requirement of which was verified) and MN42 had generation times in L broth at 37°C of 55 and 24 min, respectively. These strains were transformed with pAM1 and pKC17 and tested by cross-streaking against IPTG. Contrary to transformants derived

TABLE 2. Extent of minicell formation in *minB*^{sup} mutants[a]

Strain	Growth temp (°C)	IPTG	% Polar divisions (no. with polar divisions/no. tested)
JS327 (*min-16*)	26	−	37 (52/141)
	26	+	47 (49/104)
	37	−	54 (84/154)
JS328 (*min-20*)	26	−	0 (1/205)
	26	+	2 (6/301)
	37	−	24 (49/205)
JS329 (*min-17*)	26	−	10 (21/204)
	26	+	ND[b]
	37	−	32 (68/211)
JS330 (*min-18*)	26	−	28 (56/198)
	26	+	32 (65/203)
	37	−	21 (43/204)

[a] Strains were grown in L broth containing ampicillin and methicillin; IPTG, when present, was used at 0.5 mM.
[b] ND, Not determined, owing to division inhibition.

from MN42, transformants derived from JS412 were insensitive to IPTG. The sensitivity to DicB of MN42 was not changed when its growth rate was adjusted to that of JS412, as shown by cross-streaking of transformants against IPTG on glucose plates containing methionine, arginine, and aspartic acid. Consequently, the resistance of JS412 to DicB is not merely due to the effect of mutation *sbr-1* on growth rate.

Properties and mapping of minicell-forming mutations that confer resistance to DicB. A total of 15 different DicB-resistant mutants showing various degrees of minicell formation were isolated. The minicell formation phenotype of *min-1* mutants (1) was shown to result from a single mutation (9, 25) located close to *fadR* at 25 min, and additional minicell-forming mutants were recently isolated and shown to map at the same location (16). To determine whether our minicell-forming mutations map in *minB* and are responsible for resistance to DicB, we established the frequency of cotransduction with *fadR* by using strain RS3032 as a donor of *fadR*::Tn*10*. Four mutants (JS327, JS328, JS329, and JS330), designated *minB*^{sup}, showed the expected 60% cotransduction frequency between the MinB$^+$ and Tc^r phenotypes. In addition, MinB$^+$ transductants always regained full sensitivity to IPTG and grew poorly, with extensive filamentation, at 37°C. It may be recalled that this temperature is nonpermissive for the original strain, JSP. At least two other minicell-forming mutations did not cotransduce with *fadR*.

We next questioned whether the extent of minicell formation correlates with resistance to DicB. The percentage of polar divisions was measured in minicell-forming derivatives of strain JSP, and the residual sensitivity of these derivatives was estimated by a cross-streaking test against a lawn of 0.5 M IPTG. Similarly, mutants carrying mutations *min-1* to *min-4* and their isogenic *min*$^+$ parent, GC7237 (16), were transformed with plasmids pAM1 and pKC17 and tested. Among mutants exhibiting a high degree of minicell formation, JS327 (*min-16*) showed approximately 40% polar divisions (Table 2) and was completely resistant, but JS329 (*min-17*), which shows a comparable amount of minicell formation, was sensitive to high levels of IPTG. *min-1* and *min-4* mutants (10 to 18% polar septa; 16) were as sensitive to DicB as their *min*$^+$ parent was, while GC7246, which shows a comparable amount of minicell formation, was completely resistant. Among strains forming small amounts of minicells, GC7245 (*min-2*), which shows only 2% polar septa (16), was fully sensitive, while JS328 (*min-20*) (Table 2) was completely resistant. Taken together, these results indicate an absence of correlation between the extent of minicell formation and the ability of the mutations to confer resistance to DicB.

When the four *minB*^{sup} mutations were introduced into the *dicA*$^+$ strain JS219 by cotransduction with *fadR*::Tn*10*, minicell-forming transductants became completely resistant to DicB (expressed from a plasmid-borne gene), indicating that the *minB*^{sup} mutations alone can confer this resistance. When the original strain, JSP, was used as a recipient for cotransduction of *min-16* with *fadR*::Tn*10*, the same association between the minicell formation phenotype and resistance to IPTG-dependent induction of plasmid-encoded *dicB* expression was observed. However, the minicell-forming transductants had retained temperature sensitivity and the associated absence of visible septation. This unexpected result is discussed below.

DISCUSSION

We isolated mutants which resist the lethal effects of the division inhibitor DicB expressed simultaneously from its chromosomal copy and from a multicopy plasmid. One class of mutations, called *minB*^{sup}, maps in the minicell locus *minB* involved in the correct placement of septation sites. These mutations are of particular interest, since they may be directly relevant to the mechanism of division inhibition by DicB. Early studies (28) showed that the canonical mutation *min-1* isolated by Adler et al. (1) has a normal frequency of divisions with respect to the frequency of termination of replication. However, in mutants containing this mutation, polar sites, which are left from previous divisions, are reused just like nonpolar sites, while such reuse is strictly prevented in wild-type strains. To account for these observations, the most simple model would be that the *minB* locus codes for a factor(s) exclusively involved in inhibiting the reuse of polar sites. However, this model would fail to explain why *minB*^{sup} mutations restore normal (i.e., nonpolar) divisions in the first place. Thus, it is reasonable to propose that the normal control over the location of septa results from a balance between two antagonistic mechanisms: one of division inhibition (affecting both polar and nonpolar sites) and a second one which limits the effects of the first one and channels division to the median site. DicB would simply prevent this second mechanism from operating. Indeed, this formal model agrees well with recent studies by de Boer et al. (10, 11), who examined the effects of proteins coded for by genes of the minicell locus. According to their studies, two polypeptides coded for by the *minB* locus, MinC and MinD, cooperate to inhibit division. A third polypeptide, MinE, prevents division inhibition at internal septation sites in wild-type cells and, when overproduced, at polar sites as well (11). It is therefore conceivable that DicB prevents MinE from exerting this regulation. However, our results do not establish any simple correlation between the extent of minicell formation and the suppression of DicB effects. Thus, *min-16* completely suppresses the effects of DicB, while *min-1*, which causes a similar amount of minicell formation, does not.

Mutation *min-16*, once transferred to a nonmutagenized background, continued to suppress the effects of plasmid-encoded DicB but not the temperature sensitivity caused by mutation *dicA1*. These results suggest that *dicA* mutants

express another division inhibitor in addition to DicB and that *minB*^sup mutants were isolated as double mutants. Indirect evidence for the existence of a second mutation comes from the fact that the original *minB*^sup mutants, when made *minB*+, did not regain their full sensitivity to temperature. Direct evidence for the presence of a second division inhibition gene, *dicF*, located in the *dicB* operon, has now been obtained (F. Bouché and J. P. Bouché, Mol. Microbiol., in press).

A second, major class of mutants resistant to both plasmid-encoded DicB and *dicA1*-dependent temperature sensitivity has been analyzed. The mutation they carry, called *sbr*, maps within the gene for the beta-subunit of RNA polymerase, *rpoB*, or possibly in the nearby gene *rplL* and confers resistance to DicB in a nonmutagenized background. *sbr* mutants grow very slowly, but this does not appear to be the cause for resistance to DicB, since an *sbr*+ isogenic strain is very sensitive to the inhibitor when grown at a similar growth rate. We have obtained preliminary evidence indicating that, in a nonmutagenized background, *sbr-1* also confers resistance to the second inhibitor, DicF. Our current hypothesis is that the original *sbr* mutations correspond to a single, rare (5×10^{-5} after EMS mutagenesis) mutation which affects differentially the expression of a group of genes and possibly of genes involved in triggering septation.

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