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Novobiocin Resistance Marker in *Haemophilus influenzae* That Is Not Expressed on a Plasmid

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The plasmid pNov2, carrying a cloned chromosomal marker conferring resistance to at least 2.5 μg of novobiocin per ml, was constructed with a new *Haemophilus influenzae* cloning vehicle, pDM2. The novobiocin marker of pNov2 was not normally expressed, but in Rec$^+$ cells approximately one in 10^4 cells in a culture of a transformant became novobiocin resistant, a frequency about four orders of magnitude higher than the spontaneous mutation frequency. Variants of such cells that had lost the plasmid were also novobiocin resistant. Since Rec$^-$ cultures bearing pNov2 showed novobiocin resistance only at the normal mutation frequency, we concluded that the Rec$^+$ novobiocin-resistant transformants arose because of a rare recombination between plasmid and chromosome in which the chromosome acquired the novobiocin marker from the plasmid. Evidence is presented that novobiocin sensitivity is dominant over this particular novobiocin resistance marker.

In an earlier paper (7) we described the properties of a recombinant plasmid, pNov1, in which a chromosomal novobiocin marker of *Haemophilus influenzae* has been cloned into the plasmid RSF0885. Using the newly constructed cloning vehicle described in the accompanying article (5), we isolated another type of plasmid (pNov2) bearing a novobiocin resistance marker. Novobiocin resistance in *H. influenzae* occurs at three known novobiocin levels: to 50, 5, and 1 μg/ml (3). The high-level resistance marker consists of two closely linked mutations which separately confer resistance to 5 and 1 μg/ml. Whereas pNov1 carries this high-level resistance marker, pNov2, containing a chromosomal insert only about 1/10 as long as that of pNov1, confers resistance only up to 5 μg/ml. The present paper describes the profound difference between these plasmids, particularly with respect to their behavior in transformation. The novobiocin marker in pNov2, unlike that in pNov1, was not expressed in a cell containing the plasmid unless there was a recombination event.

MATERIALS AND METHODS

Microorganisms and media. Information on *H. influenzae* strain BC200 and mutant *rec-1*, growth media, and transformation methods is given in the accompanying paper (5). Strain Rd is a wild-type *H. influenzae* strain that contains the defective prophage present in most laboratory strains but not in BC200 (2). Voll and Goodgal (8) have described resistance to at least 2.5 and 25 μg of novobiocin per ml. Following their notation, we have referred throughout this paper to the 2.5 and 25 novobiocin markers. These are the loci described by Day and Rupert (3) as Nb_1 and Nb_2, conferring resistance to up to 5 and 50 μg of novobiocin per ml, respectively.

Isolation of pNov2. The cloning vehicle pDM2 is described in the accompanying paper (5). *H. influenzae* chromosomal DNA containing eight antibiotic resistance markers and the cloning vehicle were cut with the restriction endonuclease *Pst*I and ligated as described (5) to create a library of chloramphenicol-resistant clones. Individual clones were spotted onto the surface of agar plates containihg chloramphenicol plus one of the eight antibiotics and onto one plate containing chloramphenicol alone. The spot from one of the 144 clones of this set showed approximately 100 colonies on the plate containing novobiocin at 2.5 μg/ml. Other clones showed no evidence of novobiocin resistance. The corresponding clone from the chloramphenicol plate was regrown in chloramphenicol, but did not grow well in 2.5 μg of novobiocin per ml. A plasmid preparation made from these cells grown only in chloramphenicol showed on an agarose gel a single plasmid which upon digestion with *Pst*I gave two bands, one the size of the linearized cloning vehicle and another of approximately 0.8 kilobases. Thus, the insert of this plasmid is more than 10 times smaller than the 9.4-kilobase insert of pNov1 (7).

Surface colonies were obtained from the chloramphenicol-grown BC200 pNov2 culture, and 104 of them were transferred with toothpicks to plates containing chloramphenicol and ampicillin or chloramphenicol and novobiocin. There was no growth in ampicillin, suggesting that there had been no regeneration of the cloning vehicle in these cells, as has been observed for some originally ampicillin-sensitive clones (5). However, there were scattered colonies from each isolate on the plates containing novobiocin.

Colonies were picked from one of the isolates and were found to grow well in 2.5 μg of novobiocin per ml. This culture was grown up, frozen, and later used for making some of the plasmid preparations of pNov2.

Chloramphenicol-sensitive variants. We obtained cells without plasmids by growing the cultures for many generations without chloramphenicol and then plating them on the surface of growth medium plates without antibiotic for toothpick transfer to plates with and without chloramphenicol. Since the plasmids were stable under these conditions, about 100 to 300 colonies of each culture needed to be tested to obtain at least one chloramphenicol-sensitive variant. Loss of the chloramphenicol marker was always associated with loss of the plasmid, as checked by gel assays of crude plasmid preparations (6).

Growth measurements. The optical density at 675 nm was read in a Bausch and Lomb Spectronic 20 colorimeter.

RESULTS AND DISCUSSION

Level of novobiocin resistance of pNov2. The BC200 clone of cells containing pNov2 isolated from a novobiocin-containing plate was able to grow well in 2.5 μg of novobiocin per ml but not in 25 μg/ml, unlike a pNov1-containing strain made with the same chromosomal DNA. The fact that pNov1 was constructed with the aid of the restriction endonuclease *Pvu*II (7) but *Pst*I was used in construction of pNov2 thus indicated that there is a *Pst*I site between the two closely linked novobiocin loci on the *H. influenzae* chromosome.

Chloramphenicol-sensitive variants from the culture carrying pNov2 isolated as described above were also resistant to 2.5 μg of novobiocin per ml.

Transformation by pNov2. Competent strain BC200 exposed to a highly purified preparation of pNov2 (1.2 μg/ml) yielded 6.7×10^3 chloramphenicol transformants per ml and 3.9×10^5 novobiocin transformants per ml. The same competent cells exposed to the same concentration of purified pNov1 gave 4.9×10^6 ampicillin transformants per ml and 4.4×10^7 novobiocin transformants per ml. Thus there is much more transformation by pNov2 for novobiocin resistance than for the plasmid marker chloramphenicol resistance, analogous to the ratio of novobiocin to ampicillin resistance from pNov1. Also as with pNov1 transformation (7), there is a linear relationship between DNA concentration and the number of pNov2 transformants of both types, except at very high DNA concentrations (data not shown).

The overwhelming majority of pNov1 transformants to novobiocin resistance are without plasmids; the novobiocin marker that has been introduced by the plasmid is integrated into the chromosome (7). Ten pNov2 transformants to novobiocin resistance were isolated and found to be chloramphenicol sensitive and thus are also without plasmids.

Transformation was not nearly as efficient for pNov2 as for pNov1, a phenomenon which is not surprising in view of the relative sizes of the inserts. It is of interest that a *Pst*I digest of *H. influenzae* DNA (1) has been found to transform for the novobiocin 2.5 marker only 0.02% as well as does uncut DNA and 0.07% as well as does *Pvu*II-cut DNA (used for pNov1 construction). However, the ratio of novobiocin transformants from pNov1 and pNov2 was only about 100, implying that transformation by a small fragment is favored when it is attached to nonhomologous DNA. The size of the *Pst*I fragments obtained from chromosomal DNA digestion is 0.9 kilobases (1), close to our determination of 0.8 kilobases as the size of the *Pst*I insert of pNov2.

Growth behavior in novobiocin of pNov2 transformants selected for chloramphenicol resistance. Thirty transformants of strain BC200, three transformants of strain Rd, and ten transformants of mutant *rec-1*, all by pNov2, were put into novobiocin (2.5 μg/ml) plus chloramphenicol (4 μg/ml). All behaved like novobiocin-sensitive cells with respect to their growth. Ten of the BC200 transformants, when grown in 1 μg of novobiocin per ml, also appeared to be sensitive. A typical example is shown in Fig. 1. For comparison, 5 ampicillin transformants of strain Rd and 10 of mutant *rec-1*, all by pNov1, were treated similarly, except that novobiocin at 25 μg/ml and ampicillin at 5 μg/ml were in the medium. All of the *rec-1* pNov1 transformants behaved like novobiocin-resistant cells. The Rd pNov1 cells (Fig. 1) were of two types: either they were able to grow normally in 25 μg of novobiocin per ml or they were superficially like the pNov2 transformants and showed no evidence of novobiocin resistance, with a growth pattern in novobiocin like that of the novobiocin-sensitive strain Rd without plasmids (data not shown). The pNov1 novobiocin-sensitive transformants contained no novobiocin resistance markers, since lysates of such cells were unable to transform sensitive cells to novobiocin resistance and contain plasmids with genetic information for novobiocin sensitivity (7). However, the pNov2 novobiocin-sensitive transformants did contain plasmids bearing a novobiocin resistance marker, since crude lysates as well as purified plasmid preparations of such transformants readily transformed sensitive cells to novobiocin resistance (data not shown). Furthermore, the ratio of novobiocin to chloramphenicol BC200 transformants from purified plasmid preparations was slightly below 10^{-2}, which is similar to the ratio obtained with a purified pNov2 preparation made with cells that

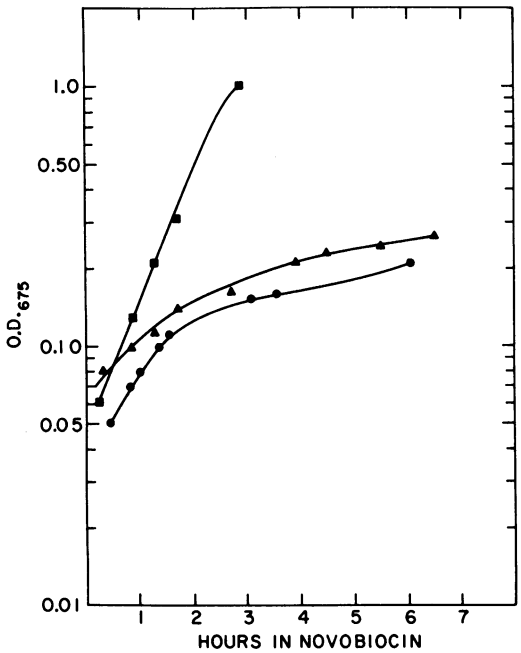

FIG. 1. Growth of strain Rd transformed to chloramphenicol resistance with pNov2 (●) or to ampicillin resistance with pNov1 (■ and ▲) and then subcultured in 2.5 μg of novobiocin plus 4 μg of chloramphenicol per ml (●) or in 25 μg of novobiocin plus 5 μg of ampicillin per ml (■ and ▲). OD_{675}, Optical density at 675 nm.

also contained a chromosomal novobiocin marker. Thus the novobiocin resistance marker was present but not expressed in the Rec^+ or Rec^- pNov2 transformants, unlike the phenotypically novobiocin-sensitive Rec^+ pNov1 transformants, which have lost the novobiocin resistance marker altogether (7). The novobiocin resistance marker in the pNov2 transformants was also not expressed in any obvious effect on DNA synthesis, since we were unable to show any difference in the kinetics of uptake of tritiated thymidine in the presence of novobiocin (2.5 μg/ml) by novobiocin-sensitive cells with or without the pNov2 plasmid (data not shown).

Selective advantage of pNov2-containing cells in novobiocin. Because of the lack of observable expression of pNov2 in transformants, it became difficult to understand what had been the selective advantage of such cells that enabled us to isolate a clone containing that plasmid.

We observed that eight pNov2 chloramphenicol transformants that had not been in novobiocin did not contain the novobiocin resistance marker in the chromosome, since chloramphenicol-sensitive variants in cultures obtained by testing 883 isolates from these transformants were all sensitive to 1 and 2.5 μg of novobiocin per ml. Three of these transformants were left in novobiocin overnight, and the culture became novobiocin resistant, as judged by eventual normal growth in novobiocin at these concentrations. Chloramphenicol-sensitive variants of these transformants were all novobiocin resistant, and thus the chromosome now contained the novobiocin resistance marker.

There could be at least two reasons why cells containing pNov2 might be more likely to acquire a chromosomal novobiocin resistance marker than would a plasmid-free cell: (i) the plasmid could recombine with the chromosome or (ii) the plasmid could alter the spontaneous mutation rate of the chromosomal DNA. To test the first idea, we measured the number of novobiocin-resistant cells in the apparently novobiocin-sensitive cultures of Rec^+ and Rec^- cells with and without the pNov2 plasmid (Table 1). In a Rec^+ culture without the plasmid, the frequency of novobiocin-resistant cells was 3×10^{-8}, reflecting the spontaneous frequency for mutation to resistance to 2.5 μg of novobiocin per ml as previously measured (4). Similar numbers were obtained with *rec-1* transformants carrying the pNov2 plasmid. However, the frequency in the Rec^+ pNov2 transformants was close to four orders of magnitude higher. Since this phenomenon was obviously dependent on the *rec-1* gene, we postulated that the chromosome of a small fraction of the Rec^+ cells had acquired the novobiocin marker from one of the pNov2 plasmids by recombination, rendering the cells novobiocin resistant.

Alteration of the spontaneous mutation rate by the plasmid apparently did not occur, since mutation to erythromycin and streptomycin resistance was the same with and without the

TABLE 1. Cells resistant to novobiocin (2.5 μg/ml) in Rec^+ and Rec^- cultures with and without a plasmid

Culture	No. of novobiocin-resistant cells/total no. of cells
Rec^+ BC200 without plasmid	3×10^{-8}
Rec^- *rec-1* transformants with pNov2	
1	1.6×10^{-8}
2	0.8×10^{-8}
3	2×10^{-8}
4	3×10^{-8}
Rec^+ BC200 transformants with pNov2	
1	1.0×10^{-4}
2	2.1×10^{-4}
3	1.0×10^{-4}
4	1.4×10^{-4}

plasmid present (data not shown). Furthermore, previous studies have shown that there is no observable difference in the mutation frequency of *rec-1* and Rec^+ cells (4). Thus, we consider that the selective advantage of Rec^+ cells containing pNov2 lies entirely in their ability to become novobiocin resistant by recombination.

Time of occurrence of recombination between pNov2 and the chromosome. In our previous study of the plasmid pNov1 (7), we obtained evidence that that plasmid recombined with the chromosome only at the time of the original transformation event rather than later during the subsequent growth of the culture. The same cannot be true of the recombination between pNov2 and the chromosome, since only about 1 in 10^4 cells apparently have acquired the novobiocin marker from the plasmid. These data suggest that the recombination takes place, on the average, when there have been about 13 replications of the genome of the transformed cell.

Dominance and recessiveness of the novobiocin 25 and 2.5 markers. There are at least two possible explanations for the lack of expression of the 2.5 marker when it is exclusively on a plasmid and not on the chromosome, although the 25 marker expresses perfectly well under similar conditions. The novobiocin 2.5 marker is smaller and is inserted into a different region of pDM2 than that of the novobiocin 25 marker in plasmid RSF0885 (7). Thus, there could be a problem with expression of the cloned marker in the smaller insert, especially if the cloned genes were separated from their normal regulatory elements or if a truncated peptide were produced by the plasmid. Alternatively, there could be a difference in the dominance of the two genes. To test the latter possibility, a *rec-1* strain with a chromosomal marker (either novobiocin 2.5 or novobiocin 25) was exposed to plasmid pNov1s, which carries novobiocin sensitivity (7), and cells were selected for ampicillin resistance. The Rec^- strain was used to avoid recombination between the plasmid and the chromosome. Ten transformants of each type were isolated, grown up in ampicillin, and then transferred to medium containing 2.5 μg of novobiocin per ml (for mutant *rec-1* carrying the corresponding marker), or 25 μg of novobiocin per ml (for mutant *rec-1* carrying the high-level marker). The results are given in Fig. 2, which shows an example of each type. Similar results were obtained for all transformants of a single type. Thus, the novobiocin 2.5 transformants were more like sensitive strains in their slow rate of growth, but they all eventually grew to saturation. On the other hand, the novobiocin 25 transformants initially seemed like sensitive cells, but the growth rate steadily increased and approached that of resistant cells (Fig. 1). One

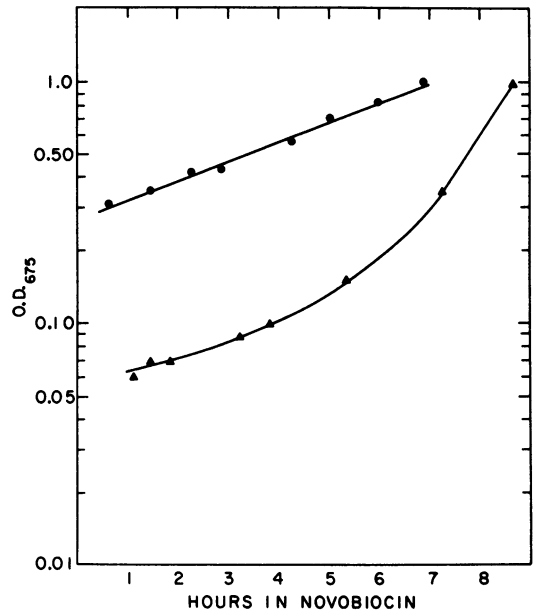

FIG. 2. Growth of the *rec-1* mutant resistant to 2.5 (●) or 25 (▲) μg of novobiocin per ml and transformed to ampicillin resistance by the plasmid pNov1s carrying information for novobiocin sensitivity. Both strains were in 5 μg of ampicillin per ml, and the concentration of novobiocin was 2.5 (●) or 25 (▲) μg/ml. OD_{675}, Optical density at 675 nm.

explanation of this phenomenon is that the average plasmid copy number may have decreased as the cells were forced to grow in novobiocin and that when the copy number was low enough, the chromosomal marker became dominant. Measurement of the copy numbers obtained from profiles of DNA labeled with [^{3}H]thymidine in CsCl-ethidium bromide showed that when the novobiocin 25 cells containing the pNov1s plasmid were grown for many hours in novobiocin, the copy number changed from the initial value of around 30 to less than 10.

Whereas this experiment did not prove that the dominance relationship is reversed in the two types of plasmid-carrying strains, it indicated that novobiocin sensitivity genes on a plasmid interfered considerably with the expression of the chromosomal novobiocin 2.5 marker and interfered only for a time with the expression of the 25 marker. The data raised the possibility that when the novobiocin sensitivity gene is on the chromosome, it might similarly interfere with the plasmid novobiocin 2.5 gene expression but not with the novobiocin 25 gene expression.

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