Molecular Cloning of the Wild-Type *phoM* Operon in *Escherichia coli* K-12

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A metastable bacterial alkaline phosphatase (Bap) phenotype is seen in *phoR* mutants, which alternately express a Bap-constitutive or -negative phenotype. The alternation is affected by mutations in the *phoM* region near 0 min. By molecular cloning of the wild-type *phoM* operon onto a multicopy plasmid and recombining onto the plasmid the *pho-510* mutation that abolishes variation, the *phoM* operon, rather than some nearby gene, was shown to control variation. Complementation tests indicated that the wild-type *phoM* allele is dominant to the *pho-510* mutation when both are in single copy, but whichever allele is present in higher copy appears as dominant when multicopy plasmids are examined. The alternating phenotypic variation of BAP synthesis was not seen in *phoR*⁺ cells with multicopy wild-type *phoM* plasmids, thus showing that the variation is associated with *phoM*-dependent Bap expression. The alternation acted at the level of *phoA* transcription; it was also *recA* independent. BAP clonal variation is phenotypically similar to *Salmonella* phase variation, which is controlled by a DNA rearrangement. No evidence was found for a DNA change near the *phoM* operon that might be responsible for the variable Bap phenotype.

Bacterial alkaline phosphatase (BAP) synthesis is normally regulated by the environmental phosphate levels. When the amount of inorganic P_i is limiting for growth, the expression of the Bap structural gene, *phoA*, is induced several hundredfold. The *phoA* gene is a member of the phosphate regulon, which includes several unlinked phosphate-starvation-inducible (*psi*) promoters (22). The *phoA*, *phoE*, *psiB(ugp)*, *psiC(ugp)*, *psiD*, and *pstSCAB* genes are involved in phosphorus assimilation (16, 22); they are induced during phosphate limitation and regulated in *trans* by the *phoBR* operon (24, 26). The *phoB* and *phoR* gene products at the protein sequence level share similarities with other proteins that are elements of two-component regulatory systems that respond to various sorts of environmental signals (12). Accordingly, PhoR may act as a phosphate sensory or transducer protein that interacts with PhoB to activate transcription.

phoB mutants are uninducible for *phoBR*-dependent *psi* promoters because PhoB is a transcription activator. PhoR has a dual regulatory role as both an activator and a repressor (22, 25), which apparently reflects its function as a sensory protein. Negative control of the phosphate regulon requires, in addition to PhoR, the *pstSCAB-phoU* operon gene products, which include components of the high-affinity phosphate-specific transport (Pst) system and the PhoU repressor. Whereas phosphate transport via a low affinity Pit system is sufficient to maintain normal rates of growth when the inorganic P_i levels are in excess, a functional Pst system and PhoU are required for repression of *phoBR*-dependent *psi* promoters (22). The *pstSCAB* and *phoU* proteins probably interact with PhoR, in a manner that communicates to PhoR the environmental P_i levels; the *pstSCAB-phoU* genes

are without effect on the phosphate regulon in the absence of a functional *phoR* gene product (19).

The activation of several *psi* promoters no longer responds to phosphate limitation in null-type *phoR* mutants. However, the *phoA* and other *phoBR*-dependent *psi* promoters are still expressed, and their transcription still requires PhoB. In *phoR* mutants, these promoters are also regulated by the unlinked *phoM* gene(s), which is without effect on *psi* gene transcription in *phoR*⁺ cells (22). Interestingly, two gene products in the *phoM* operon (1) share similarities at the protein sequence level with PhoB and PhoR and elements of other two-component environmental regulatory systems (12; C. Ronson, personal communication). No function has yet been assigned to the *phoM* operon genes, except in the control of the phosphate regulon in *phoR* mutants (25).

It was thought until recently that *phoR* mutants synthesized BAP constitutively; this expression required PhoM, which could substitute for PhoR as an activator (22, 25). However, earlier studies usually used bacteria with the *pho-510* mutation that maps near 0 min, i.e., in the vicinity of the *phoM* operon. (The *phoBR* operon maps near 9 min.) The *pho-510* allele is responsible for the Bap-constitutive phenotype of some *phoR* mutant strains (19, 20). This mutation apparently arose over 40 years ago by X-ray mutagenesis in the formation of the *Escherichia coli* K-12 strain 58F⁺. The presence of the *pho-510* allele in this ancestral K-12 strain led to its being widely distributed. Its presence in numerous common laboratory K-12 strains is why Bap constitutivity was thought for so long (20) to be the *phoR* mutant phenotype.

An unusual pattern of *phoA* gene regulation is seen in *phoR* mutants that are otherwise wild type. Such mutants alternately express a Bap-constitutive or -negative phenotype. The alternating pattern is called Bap clonal variation; cells switch between expressing one phenotype and the other when grown on a neutral (noninducing) medium (19, 20). The alternation acts at the transcriptional level of the

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phoA gene; it is also highly regulated by the growth media (19, 20; unpublished data).

We know that it is the wild-type *phoM* region rather than a mutant form of it which is responsible for the Bap clonal variation phenotype. This was inferred by examining the pedigrees of various *E. coli* K-12 lines. Strains BD792, EMG2, MG1655, W1485, and W3110, which are closely related to wild-type K-12 and include unmutagenized lines, acted as donors for the particular *phoM* region that led to the Bap clonal variation phenotype. It is also most likely that all strains that donated a *phoM* region for constitutivity in *phoR* mutants have the same (*pho-510*) allele in the *phoM* region. This was inferred from the ancestry of many constitutive donors which could be traced to strain 58F⁺. This strain donated an allelic form for constitutivity, but its parent and other strains related to its parent via a different lineage did not. Furthermore, strains that donated an allele for constitutivity but whose pedigrees were less certain are likely to have acquired the *pho-510* allele in matings. Several popular Hfr strains, including HfrC and HfrH, which donate the *phoM-thr* 0-min region as an early marker, are themselves 58F⁺ descendants and contain the *pho-510* constitutive allele (20; unpublished data). (The wild-type *phoM* operon was previously identified as the *pho-499* allele [20]; this designation is no longer in use.)

The *phoM* gene(s) was cloned (2, 9, 10, 15, 22; unpublished data) before the Bap clonal variation phenotype was discovered (17, 19). When a multicopy plasmid containing the wild-type *phoM* region was used to complement Bap-negative Δ *phoM phoR* mutants, we found that the *phoA* gene was not expressed constitutively. Instead, the transformants displayed a metastable phenotype; they alternated between Bap-constitutive and -negative phenotypes. The metastable behavior with *phoM*(wt) plasmids is described in this paper. It was a consequence of these studies which eventually led to the discovery of the Bap clonal variation phenotype in *phoR* mutants (17, 19). It is shown that Bap variation in *phoR* mutants containing *phoM*(wt) plasmids does not appear to be associated with a DNA change near the *phoM*(wt) operon, nor with a mutational change in the chromosome. A new role for the *phoM* operon in gene expression is also proposed.

MATERIALS AND METHODS

Bacterial and plasmid strains. All bacteria used to study BAP regulation in this paper descended from *E. coli* K-12 strain XPh1a (25) in a series of P1 crosses. The Δ *phoM phoR* mutant BW2091 [Δ *lac-169* Δ (*trpR phoM dye thr chlG*)93 *phoR68 rpsL267 crp-72 thi*] was identified as a Bap-negative mutant that was selected for the loss of a Tn10 insertion clockwise of the *thr* operon (23). The *pho-510*, *phoM451*, *phoM453*, and *phoM*(wt) alleles were crossed into strain BW2091 by selecting Thr⁺ transductants with P1 grown on strains BW3912, BW499, BW304, and BW3414 (18), respectively. The complementation tests were done with *recA1* derivatives.

The plasmid pSE356 was provided by S. Elledge. The strain GM119 (*dam-3*; 11) was provided by J. Brookes, RR1 with pRK2013 (4) was provided by L. Csonka, and EST945 was provided by the late E. S. Tessman.

Media, chemicals, enzyme assays, and recombinant DNA methods. The media, chemicals, enzyme assays, and recombinant DNA methods were as described previously (18–22). XP and XG are the blue dyes used to detect BAP and β -galactosidase activities, respectively. Enzyme units are

nanomoles of product made per minute. *BclI* and *Clal* digestions were done on plasmid DNAs that were isolated from a *dam-3* host.

Construction of *recA* transductants. The heterozygous strains BW9301 (*recA1*/ λ *recA*⁺) and BW9355 (*recA1 srlC300::Tn10*/ λ *recA*⁺) were used to make efficient Plc lysates for transduction of the *recA1* allele by selecting Srl⁺ or tetracycline-resistant (Tc^r) transductants, respectively.

Transfer and curing of ColE1-based plasmids. The *trans*-acting transfer plasmid pRK2013 (4) was mated from RR1 into the multiply auxotrophic and drug-sensitive strain BW358 [*ilv-1 his-29*(Am) *trpA9605 trpR tsx ara malT phoM*(wt)]. Kanamycin-resistant (Kan^r) exconjugants were tested for recipient markers. Strain BW358(pRK2013) was routinely used in two ways: in conjugal transfer of non-self-transferable ColE1 plasmids in tripartite matings in which drug-resistant prototrophs were selected, and in incompatibility curing of ColE1 plasmids in bipartite matings in which Kan^r prototrophs were selected.

Patch matings were done on a complex (tryptone-yeast extract; TYE) agar for 3 h, after which donors, recipients, and mixed patches were streaked onto a selective medium. Exconjugants were purified once and tested for relevant markers. The transfer of pBR322 clones with pRK2013 requires the *cis*-acting *bom*⁺ region of ColE1, which maps between the tetracycline resistance gene and replication origin of pBR322 (28). Matings were used to screen new plasmids and to verify which phenotypes were plasmid associated.

Molecular cloning of the *phoM*(wt) region. Strain BW2246 [W3110 *thr::lacZY*(Mu d1) *phoM*(wt) *rpsL* Δ *lac-169*] chromosomal DNA was digested with *PstI* and ligated with *PstI*-cut pBW2 by using a version of the Mu-*lacZ* direct cloning vector (21) with the pUC8 polylinker region. Ampicillin-resistant (Amp^r) HB101 transformants were selected. Several chimeric plasmid DNAs were analyzed and shown to have *PstI*, *HindIII*, and *EcoRI* restriction fragments which corresponded in size to chromosomal DNA fragments by Southern blot hybridization (data not shown). One resultant plasmid was named pBW20. Its 6.2-kilobase (kb) *EcoRI*-*HindIII* fragment was purified on agarose, electroeluted, and ligated with suitably restricted pSE356, a λ shuttle vector (S. Elledge, personal communication). The plasmid pSE356 was digested to completion with *EcoRI* and partially with *HindIII*. Ligated DNAs were used to select Kan^r BW2091 transformants. Several plasmid DNAs were purified, and their restriction patterns were analyzed. All plasmids with the 6.2-kb donor fragment showed the same Bap phenotype in *phoR* mutants. One plasmid was named pMW1(Var⁺) and studied further.

Two plasmids that led to a Bap-constitutive (stable; Var⁺) phenotype were made from pMW1(Var⁺) transformants of *recA*⁺ bacteria with various chromosomal *phoM* regions. One plasmid originated in a pMW1(Var⁺) transformant of the Bap-negative mutant BW8660 (*phoM453 pho-510 phoR68*). Such transformants showed the variable Bap phenotype but occasionally gave rise to constitutive colonies that no longer showed variation. The plasmid pMW1(Var⁺)₁ was identified in one such stably constitutive colony, in which constitutivity was associated with the plasmid, as determined in transformation tests of strain BW2091 (Δ *phoM phoR68*). The plasmid pMW1(Var⁺)₂ was deliberately made by transforming strain BW8882 (*pho-510 phoR68*) with pMW1(Var⁺) and then treating it with UV irradiation to enhance recombination, isolating plasmid DNA, and testing the DNA by transforming strain BW2091 to Kan^r on a

complex BAP indicator (TYE-XP) agar. Plasmid DNA was isolated from dark blue colonies that appeared to be unselected and verified by retransforming strain BW2091 and testing transformants for their Bap phenotype, as described elsewhere (19). When the plasmid DNAs were characterized with restriction enzymes, no differences in the DNA restriction patterns for pMW1(Var⁺) and its Var⁻ derivatives were seen. The plasmids were compared side-by-side on agarose gels after single digestions with *AluI*, *HaeII*, and several 6-base-pair recognition enzymes (data not shown). It is likely that both pMW1(Var⁻)₁ and pMW1(Var⁻)₂ arose by recombination of the chromosomal *pho-510* allele onto pMW1.

RESULTS

The Bap clonal variation phenotype and the wild-type *phoM* operon. The studies reported in this paper were mostly done several years ago and led to the discovery of the Bap clonal variation phenotype in *phoR* mutants. While this work was in progress, four other groups reported the molecular cloning of the *phoM* operon (2, 9, 10, 15, 22), without mentioning a metastable Bap character. Since our *phoM* plasmids led to a metastable phenotype and no such instability was noted elsewhere, we examined several *phoM* plasmids side-by-side. The studies of the various *phoM* plasmids and the bacteria from which they were made (19) eventually led us to conclude that differences exist in the *phoM* operon among common laboratory *E. coli* K-12 strains with respect to the Bap phenotype in *phoR* mutants.

Strains that show the Bap clonal variation phenotype have the *phoM*(wt) chromosomal region and are termed variable (Var⁺); *phoR* mutants with the *pho-510* mutant form of the *phoM* region are constitutive and nonvariable (Var⁻; 19, 20). Although P1 transductional crosses showed that the *pho-510* mutation(s) lies near the *phoM* region (20), evidence showing that it mapped within the *phoM* operon was lacking. The Δ (*phoM-thr*)93 mutation that was used to characterize this region extends beyond the *trpR* gene to the left and the *dye*, *thr*, and *chlG* genes to the right of the *phoM* operon (23; data not shown). Although three-factor crosses showed that the *pho-510* mutation probably mapped to the left of the *thr* gene, more accurate mapping was difficult because of the nature of the Bap phenotype itself.

The alternating Bap phenotype is clearly seen in *phoR* mutants that are otherwise wild type when cells are grown on a noninducing (neutral) BAP indicator medium. Since it is now evident that the wild-type *phoM* operon leads to the Bap clonal variation phenotype, the *pho-499* designation, which was previously assigned to the allele in variable strains (20), will no longer be used. Instead, the wild-type *phoM* operon is either given no specific designation or it is called the *phoM*(wt) operon. Since several mutants, including ones with the *phoM451* and *phoM453* alleles, were isolated from a strain containing the *pho-510* mutation (20), the resultant mutants are doubly mutated in the *phoM* region.

Several attempts were made to identify a plasmid containing the *phoM* gene by complementing the Bap-negative Δ *phoM phoR* mutant BW2091 (23). While some plasmids could restore BAP synthesis, the plasmids did not lead to a constitutive (stable) phenotype. In retrospect, this was because we had cloned the *phoM*(wt) chromosomal region that led to a Bap variable phenotype. The metastable Bap phenotype was unexpected because our earlier studies on *phoM*-dependent *phoA* gene expression (25) dealt with bacteria containing the *pho-510* mutation for constitutivity (20).

The metastable Bap phenotype in our transformants led us to reexamine earlier experiments in which we had previously noticed an instability of the Bap phenotype in some *phoR* mutant strains that we had made with P1 crosses (17, 19; unpublished data). As a consequence, we found that *phoR* transductants of some *E. coli* K-12 strains showed a constitutive phenotype, whereas *phoR* transductants of other K-12 strains showed the metastable Bap phenotype (19). Whether the transductants displayed the constitutive (stable) or metastable Bap phenotype depended upon a genetic difference (the *pho-510* allele) that mapped in the *phoM* region (20). As described below, the *pho-510* mutation probably maps within the *phoM* operon. Since some laboratories cloned the *pho-510* mutant form of the *phoM* operon, those plasmids did not lead to a metastable Bap phenotype.

While this study was in progress, the complete nucleotide sequence of the *phoM* gene was determined. The *phoM* gene is in an operon that encodes four open reading frames (ORFs; 1). Although *phoM* mutations that abolish BAP synthesis map in ORF3 (1; M. R. Wilmes and B. L. Wanner, unpublished data), no phenotype exists for mutations in the other ORFs. The *pho-510* mutation gives another phenotype. Until gene functions are established for this chromosomal region, "*phoM* operon" refers to the operon containing the *phoM* gene (ORF3; 25). Other genes in the operon are identified by their location, i.e., ORF1, 2, or 4.

Complementation tests with the TrpR⁺ Thr⁺ plasmid pRPG1. The *phoM* gene maps between the *trpR* and *thr* genes (23). Since the plasmid pRPG1 (7) carries this region, it was tested for complementation of the Bap-negative Δ *phoM phoR* mutant BW2091. If pRPG1 carried the *phoM* gene, transformants were expected (at the time) to show a Bap-constitutive phenotype. Although some pRPG1 chloramphenicol-resistant (Cm^r) transformants were constitutive, many were not. Also, the constitutive ones appeared to be extremely unstable and gave rise to frequent Bap-negative colonies, apparently without a loss of the plasmid. In some cases, greater than 20% of the cells in a streak of a constitutive transformant were Bap negative. Colonies with colored sectors were also commonly seen. When the plasmid DNAs from several Bap-constitutive colonies were used to retransform strain BW2091, no stably constitutive transformants were seen. This made it unlikely that the instability was due to multiple plasmid types in the transformants. Because pRPG1 transformants had an apparently unstable Bap phenotype, a new plasmid was made by cloning the *phoM* operon directly from the chromosome.

Molecular cloning of the wild-type *phoM* operon with a *thrA::Mu d1* (Lac⁺) insertion nearby. The vector pBW2 (21) has the carboxyl end of the *bla* gene immediately distal to its *PstI* site. Since the ligation of *PstI*-digested chromosomal DNA of a Mu d1(*bla lacZ*) insertion with pBW2 DNA restores the *bla* gene, Amp^r chimeric plasmids are selectable. These plasmids have the *bla* amino terminus and simultaneously acquire other Mu d1 sequences, including the *lacZ* gene, the chromosomal fusion joint, and DNA adjacent to the nearest chromosomal *PstI* site. The Mu-*lac* direct cloning vector pBW2 (21) was made to allow the cloning of the *phoM* region by using an appropriately oriented *thr-lacZ* (Mu d1) insertion nearby; it was also useful in the direct cloning of other Mu-*lacZ* transcriptional and translational fusions (21; unpublished data).

Strain BW2246 has a Lac⁺ *thr::Mu d1* insertion near the *phoM*(wt) operon (unpublished data). Since the *thr* operon is transcribed clockwise, the *phoM* gene(s) maps within its upstream region (Fig. 1). The *phoM* gene was selectively

FIG. 1. Physical structure of the *trpR phoM dye thrA* chromosomal region. The thickened lines show the DNA regions that are sequenced. Plasmid pBW20 was made by cloning into the vector pBW2 (21) the DNA to the right of the *PstI* site (see text). The distances on the right of the *PstI* site were determined in this laboratory and, distances to the left were determined by R. Somerville (personal communication). Only reference sites are shown. The DNA sequence for the *trpR* region is from references 6 and 14 and R. Somerville (personal communication), the ORF83-*ileR* region is from ref. 29, the region from the *EcoRI* site into ORF4 is from reference 1, ORF4 through the *dye* gene is from reference 5, and the *thrA* gene is from ref. 8. The *dye* gene is the same as *cpxC*, *fexA*, *msp*, or *sfrA* gene (unpublished data cited in reference 2).

clonable as a *PstI* fragment. To do this, strain BW2246 chromosomal DNA was isolated, digested with *PstI*, and ligated with *PstI*-cut pBW2. Amp^r transformants were selected and analyzed as described in Materials and Methods. One was retransformed into the Bap-negative Δ *phoM phoR* mutant BW2091 and named pBW20.

Transformation studies with the *phoM*(wt) plasmids pRPG1 and pBW20. The plasmid pBW20, like pRPG1, also gave constitutive transformants that segregated Bap-negative ones. Since *phoM* plasmids from two independent sources appeared to be unstable, further studies were done to examine the nature of the instability itself. It was considered that the medium was deleterious to constitutive transformants and negative colonies were being selected. However, both plasmids gave a similar proportion of Bap-constitutive and -negative colonies whether transformants were selected on a complex (TYE) or glucose minimal morpholinepropane-sulfonic acid (MOPS) agar with an appropriate drug. Also, in the case of the plasmid pBW20, which is both Amp^r and Tc^r, no difference was apparent in the Bap phenotype whether ampicillin, tetracycline, or both antibiotics were in the selective media.

To define the basis for the instability of the Bap phenotype in the pBW20 transformants, broth cultures were made of several Bap-constitutive and -negative colonies. Their plasmid DNAs were isolated and analyzed, with the expectation that a mutation (such as a deletion) in the plasmid would be found that was responsible for the different Bap phenotypes. However, no discernible difference in the plasmid DNA fragment sizes was revealed with restriction enzymes (data not shown). The frequent formation of deletions in the plasmid also seemed unlikely because there was only an infrequent loss of its Lac⁺ character, which was due to the *thrA-lacZ* fusion that was cloned on the same *PstI* fragment. Since no gross DNA rearrangements were seen, the effects of the plasmid DNAs were examined after selection for Amp^r transformants of strain BW2091. Unexpectedly, the plasmid DNAs from either Bap-constitutive or -negative colonies gave mixtures of both transformant types. It was tentatively concluded that if a DNA change were responsible for the instability, then the change must be a reversible one.

A *recA*-dependent recombination event could cause a reversible DNA rearrangement. Also, depending upon its molecular nature, such a change could have gone undetected above. To determine whether the instability of the Bap phenotype was *recA* dependent, the plasmids pRPG1 and

pBW20 were transformed into a *recA* BW2091 (Δ *phoM phoR*) mutant. Both transformant types were seen, regardless of the Bap phenotype of the plasmid DNA donor (see below). Also, there was no obvious change in the frequency for loss of the Bap-constitutive phenotype in the *recA* transformants. Both transformant types also were seen when pBW20 plasmid DNAs were reisolated from a few *recA* transformants and used again to transform a *recA* BW2091 mutant. We concluded that the metastable Bap phenotype was *recA* independent.

Bidirectional clonal variation of BAP synthesis in *phoM*(wt) plasmid transformants. When several Bap-constitutive and -negative transformants of the *recA* Δ *phoM phoR* mutants in the above experiments were purified and tested, it was discovered that both transformant types appeared to be unstable. That is, not only did the Bap-constitutive transformants give rise to frequent Bap-negative ones, but also the Bap-negative transformants gave rise again to Bap-constitutive ones. The alternating pattern for the metastable Bap phenotype occurred at a high frequency and repeatedly in both directions through several consecutive cycles of colony purifications. A similar pattern of variation was seen whether the transformants were grown on complex (TYE) or glucose minimal agar, with an appropriate drug to prevent loss of the plasmid.

The pattern of variation was similar for pRPG1 and pBW20 transformants. Figure 2 illustrates the alternation that is observed. In panel A, the colonies were derived from a single pRPG1 transformant of a *recA*⁺ strain; in panel B, the colonies are derived from a single pBW20 transformant of a *recA* mutant. Enzyme assays of colonies showed that the darkly stained (blue) transformants made 10- to 50-fold more enzyme than the lighter colored (pale blue) ones.

Association of the metastable Bap phenotype with *phoM*-dependent BAP synthesis. Experiments were done to determine whether the alternations for BAP synthesis in the *phoM*(wt) plasmid transformants occurred at the level of *phoA* transcription. This was done because the *phoM* gene affects *phoA* transcription (25). To test this, the plasmid pRPG1 was transformed into a Δ *phoM phoR* mutant with the *phoA4-lacZ* transcriptional fusion (27) in place of the *phoA* gene on the chromosome. A bidirectional alternating pattern of *lacZ* expression was seen. Hence, we concluded that the variation in BAP synthesis reflects an alternation in the transcription of its structural gene *phoA*. (The plasmid pBW20 was not similarly tested because its transformants are Lac⁺ because of the *thr-lacZ* fusion on it.)

In *phoR*⁺ bacteria, BAP synthesis is regulated by phosphate and no effect of the *phoM* gene is apparent (22, 25). To test the effect of the *phoR*⁺ gene on the metastable Bap phenotype, pRPG1 and pBW20 were transformed into an otherwise isogenic *phoR*⁺ strain. The transformants made no BAP on complex (TYE) agar (which contains an excess amount of phosphate). That is, BAP synthesis was repressed and no effect of the plasmid was seen. The transformants also showed normal induction of BAP synthesis in response to changes in the medium phosphate level (data not shown). To verify that the plasmids still conferred variability, their DNAs were reisolated from the *phoR*⁺ transformants and used to retransform strain BW2091. The resultant DNAs gave both transformant variant types. These data corroborate the hypothesis that the alternating Bap phenotype in transformants containing the *phoM* plasmids pRPG1 and pBW20 is associated with *phoM*-dependent expression of the *phoA* gene.

Searches for a DNA change associated with Bap clonal

FIG. 2. Bap clonal variation in strains with wild-type *phoM* plasmids. Transformants of Bap-negative bacteria (XPh1a Δ *phoM* *phoR68 rpsL267 crp-72 thi*) were grown on a complex BAP indicator (TYE-XP) agar with an appropriate drug. Bap-negative and -constitutive clonal variants were streaked on the top and bottom areas of the petri dishes, respectively. The darker colonies are stained blue with XP and synthesized about 20-fold more enzyme than the lighter colored ones. (A) Strain BW2867(pRPG1); (B) strain BW2091 *recA1*(pBW20).

variation. Three kinds of experiments were done to determine whether a DNA change (13) was associated with the variable Bap phenotype.

(i) Plasmid pBW20 DNAs were compared physically to test for gross structural changes. To do this, the DNAs were isolated from several Bap-constitutive and -negative variant colonies. No differences were detected when they were analyzed on agarose gels after single or double digestions with *Bcl*I, *Cla*I, *Eco*RI, *Hind*III, *Pst*I, and *Sal*I (data not shown). Also, no evidence was seen for multiple plasmid species in the transformants. It should be noted that in the experiments described above, the DNAs were isolated from broth cultures made from colony transformants. In this experiment, the plasmid DNAs were purified directly from stained colonies on BAP indicator media. This was done because broth cultures tended to have a mixed population of variant types. However, similar results were found; i.e., no gross structural change in the DNA was detected in either set of experiments.

(ii) Plasmid pBW20 DNAs were phenotypically tested in transformation experiments because a DNA rearrangement could have gone undetected in the physical analyses. If a DNA change in the *phoM* region were responsible for the alternating phenotypes, then plasmid DNAs from different variant colony types would, presumably, give rise to transformant populations that correspond in their Bap phenotypes to the colony from which the DNA was isolated. The same plasmid DNAs that were physically analyzed above were used to retransform the Bap-negative Δ *phoM phoR* mutant BW2091. Amp^r transformants were selected on a complex BAP indicator (TYE-XP) agar. Twelve plasmid DNAs from Bap-constitutive and -negative colonies gave similarly mixed populations of transformant types. These data strongly imply that a change in the *phoM* plasmid DNA is not responsible for the different Bap phenotypes.

(iii) The following experiment was done to determine whether a change in the chromosome might be associated with the alternating Bap phenotypes. A chromosomal mutation could affect the expression of the *phoM* gene on the plasmid, which in turn affects *phoA* transcription. To test this possibility, several pBW20 transformants were cured of the plasmid. Spontaneously cured cells were identified as Lac⁻ (white) colonies on Lac indicator (TYE-XG) agar lacking an antibiotic. The white colonies had predominantly lost the plasmid, as indicated by simultaneous loss of the Amp^r and Tc^r phenotypes. (The plasmid pBW20 is Lac⁺ because of the *thr-lacZ* fusion; it is also Amp^r and Tc^r.) The cured cells were Bap negative, as expected. Twelve independently cured colonies, including six each from Bap-constitutive and -negative variant transformants, were made competent and retransformed with pBW20. All retransformed colonies gave similarly mixed populations of variant types, regardless of the phenotypic history of the cells. Also, the transformants again showed the metastable Bap phenotype. These data argue against a mutational change in the chromosome as the molecular basis of the variation.

Comparisons of various *phoM* plasmids. Since our *phoM* plasmids gave transformants with a metastable Bap phenotype (and other investigators did not report an instability with their plasmids), a comparison was made with various *phoM* plasmids from other sources (2, 10, 15). The plasmid pBW20, its derivative pMW1(Var⁺), pRPG1 (7), and pRB1 (2) gave the metastable Bap phenotype in strain BW2091 (Δ *phoM phoR*) transformants. However, the plasmids pJP72, pJP86 (15), and pTHR32 (10) did not. Instead, the latter set of DNAs yielded Bap-constitutive (stable) transformants. However, the restriction patterns for the *phoM*

region in these plasmids are similar (2, 7, 10, 15; data not shown). DNA hybridization was used to determine whether the same or different genes were actually cloned. Southern blotting with radiolabeled pBW20 DNA as probe showed that all plasmids except pJP86 have a 2.6-kb *Kpn*I fragment in common, which includes the *phoM* gene (Fig. 1), and the plasmid pJP86 has a larger homologous fragment, as predicted by its construction (15). The *Sal*I and *Eco*RI-*Hind*III *phoM* fragments of the predicted sizes also hybridized, as well as homologous vector fragments (data not shown).

The *phoM* plasmid phenotypes were clearly different: some produced a metastable Bap phenotype, whereas others gave a Bap-constitutive (stable) phenotype. Several hypotheses were considered to explain this apparent paradox. The results above showed that the two phenotypes could not be explained by the hypothesis that different genes were cloned. The different phenotypes did not appear to be associated with the plasmid replicon nor with the plasmid copy number. The plasmid pRPG1 (7) that shows variation and the plasmids pJP72 and pJP86 (15) that do not were made with the vector pACYC184; the plasmid pTHR32 (10) that does not show variation was made with the mini-F vector pMF3; and the plasmids pRB1 (2) and pBW20 that show variation were made with pBR322. As described below, the different phenotypes were due to which chromosomal DNAs were used in the molecular cloning of the *phoM* region.

Bap clonal variation, the wild-type *phoM* operon, and the *pho-510* mutation. We had previously noticed that strain W3110 and MC4100 transductants which received a *phoR* mutation in P1 crosses produced constitutive colonies that were extremely unstable, giving rise to frequent negative clones on BAP indicator agar (B. L. Wanner, unpublished data). A reexamination of those transductants revealed that they, like the metastable *phoM* plasmid transformants described in this paper, showed bidirectional clonal variation for BAP synthesis (19). Furthermore, the reason why certain *phoR* transductants were metastable and others were constitutive (stable) was a mutation (*pho-510*) that mapped in the *phoM-thr* region (20). Therefore, experiments were done to determine whether the different Bap phenotypes for the various *phoM* plasmids were attributable to a genetic difference among strains in the *phoM* operon.

The *phoM-thr* region was tested for the presence of the *pho-510* mutation in the various bacteria that had been used as DNA sources to clone the *phoM* gene(s). These data, which were previously reported (20), are summarized in Table 1. The *pho-510* mutation is in strains CS520, KL702 (CGSC4320), and PC0031(H1075) that were used elsewhere to clone the *phoM* region. Strain CS520 was used to make the *E. coli* K-12 bank (3), which had been used to make the plasmid in reference 9 (which was unavailable for this study). Strain KL702 was used to make pTHR32 (10), and strain PC0031 was used to make pJP72 and pJP86 (15). Importantly, the pTHR32 DNA was later used to subclone the *phoM* operon for its DNA sequencing (1). These plasmids each led to a Bap-constitutive (stable) phenotype, which is consistent with their carrying the *pho-510* mutation. However, the wild-type *phoM* allele that led to the Bap clonal variation phenotype is in strains MC4100 and W3110 (20), which were used to make the plasmids pRPG1 (7), pRB1 (2), and pBW20. We concluded that the *phoM* plasmid phenotype depends on the DNA source used to make the plasmids. The *phoM* plasmids made from bacteria with the wild-type *phoM* operon lead to the Bap clonal variation phenotype, whereas similar plasmids made from mutants with the *pho-510* allele show a Bap-constitutive phenotype.

TABLE 1. Characteristics of various *phoM* plasmids

Plasmid	Source	Bap phenotype ^a	Chromosomal DNA source ^b	Chromosomal allele ^c
pRPG1 ^d	R. Gunsalus (7)	Variable	MC4100	<i>phoM</i> (wt)
pRPG1	R. Buxton (2)	Variable	MC4100	<i>phoM</i> (wt)
pBW20	This paper	Variable	W3110	<i>phoM</i> (wt)
pMW1	This paper	Variable	Subclone of pBW20	<i>phoM</i> (wt)
pRB1	R. Buxton (2)	Variable	Subclone of pRPG1	<i>phoM</i> (wt)
pTHR32	A. Nakata (10)	Constitutive	KL702	<i>pho-510</i>
pJP72	J. Tommassen (15)	Constitutive	PC0031	<i>pho-510</i>
pJP86	J. Tommassen (15)	Constitutive	Subclone of pJP72	<i>pho-510</i>

^a Strain BW2091 transformants were tested as described in the text.

^b Plasmids pRPG1 and pRB1 (2, 7), pTHR32 (H. Shinagawa, personal communication), and pJP72 (J. Tommassen, personal communication) have been discussed previously.

^c *phoM*(wt) represents the wild-type *phoM* operon that was previously called *pho-499* (20). The *pho-510* mutant form of the *phoM* operon is responsible for the Bap-constitutive phenotype in *phoR* mutants.

^d This plasmid was tested from two sources.

Construction of nearly identical *phoM*(wt) and *pho-510*(Var⁻) plasmids. Figure 1 shows the physical structure of the *trpR-phoM-dye-thr* region. The plasmid pBW20 has DNA to the right of the *Pst*I site, and pMW1(Var⁺) has the 6.2-kb *Eco*RI-*Hind*III fragment. If the *pho-510* mutation lies within the *phoM* operon, it should be possible to recombine it onto the plasmid pMW1(Var⁺). Two such plasmids, pMW1(Var⁻)₁ and (Var⁻)₂, were independently made as described in Materials and Methods. Both plasmids behaved identically; they also gave similar data to that given by the *phoM* plasmids described above that we argued are likely to contain the *pho-510* mutation. Since the *Eco*RI site lies about 0.5 kb upstream of the translation start for ORF1 in the *phoM* operon, the *pho-510* mutation probably lies within the *phoM* operon or the 1.7-kb downstream sequence. However, in another study the *pho-510* mutation was mapped to the left (upstream) of a Mu d1 insertion in the *phoM* gene (ORF3; B. L. Wanner, M. R. Wilmes, and D. C. Young, unpublished data). We conclude that the most likely location for the *pho-510* mutation is within the *phoM* operon; it probably maps in ORF1 or ORF2.

BAP synthesis in *phoM* plasmid transformants. The amounts of enzyme in the Bap-constitutive and -negative variants differ 64-fold for pBW20, about 4.6-fold for pMW1, and 47-fold for pRPG1 transformants (Table 2). The reason for the lower ratio for pMW1 transformants is unclear. Both variant types for pMW1(Var⁺) made intermediate levels of enzyme when compared with the other transformants, however. An intermediate enzyme level could result if the cells show a very high frequency of variation because then both variant types would contain a high proportion of the opposite clonal cell type. There could be other reasons as well. For instance, quantitative differences were sometimes attributable to different batches of growth media. Although all variants in a particular experiment were usually grown on the same batch of medium, this was not done in this case because of the different drug selection used. The incubation time is probably not a factor because both variant types made more enzyme upon prolonged incubation, and the relative specific activities did not change (Table 2; compare rows 2 and 4, 3 and 5, 6 and 8, and 7 and 9).

When the Var⁻ *phoM* plasmids pMW1(Var⁻), pJP72, pJP86, and pTHR32 were examined, all transformants made 110- to 180-fold more enzyme than the Δ *phoM phoR* mutant without a plasmid (Table 2; compare rows 10 and 13 to 15 with row 1). A vector plasmid lacking the *phoM* region did not affect the amount of BAP made (data not shown). It should be noted that isogenic *phoR* mutants with a chromo-

somal *pho-510* region made about 70 enzyme units per an optical density at 420 nm of cells (20), which is about twofold higher than the amounts made in the transformants. We conclude that there is no major effect of the plasmid copy number on the amount of BAP made. However, there may exist an effect of plasmid copy number on variability, as described below.

Complementation tests within the *phoM* region. The wild-type *phoM* and *pho-510 phoM* operons were tested for dominance. This was done by transforming the plasmids pBW20(Var⁺), pJP72(Var⁻), pTHR32(Var⁻), pMW1(Var⁺), pMW1(Var⁻)₁, and pMW(Var⁻)₂ into *recA phoR* mutants containing *phoM451*(Am), *phoM453*, *pho-510*, or *phoM*(wt) alleles on the chromosome. All transformants containing a plasmid with the *phoM*(wt) region showed a variable Bap phenotype, suggesting that the *phoM*(wt) allele was dominant. However, all *pho-510 phoM* plasmids conferred a Bap-constitutive phenotype, even in bacteria with a

TABLE 2. BAP synthesis and variation in Δ *phoM phoR* mutants with various *phoM* plasmids^a

Plasmid ^b	Bap phenotype	Colony age (h) ^c	BAP sp act ^d	
			Mean	Range
None	Negative	24	0.2	0.2–0.3
pBW20(Var ⁺)	Bap ⁻ variant	24	0.4	0.2–1.1
	Bap ⁻ variant	48	1.7	0.8–3.3
	Bap ⁺ variant	24	25.6	20.0–34.6
	Bap ⁺ variant	48	109.3	92.0–135.5
pMW1(Var ⁺)	Bap ⁻ variant	24	1.4	0.8–1.7
	Bap ⁻ variant	48	4.0	1.8–6.7
	Bap ⁺ variant	24	6.0	4.9–6.8
	Bap ⁺ variant	48	19.7	13.2–28.3
pMW1(Var ⁻) ₁	Constitutive	24	30.4	25.8–35.9
pRPG1(Var ⁺)	Bap ⁻ variant	48	1.4	0.9–1.7
	Bap ⁺ variant	48	66.3	35.0–91.0
pJP72(Var ⁻)	Constitutive	24	36.3	30.1–45.0
pJP86(Var ⁻)	Constitutive	24	25.5	20.3–37.3
pTHR32(Var ⁻)	Constitutive	24	22.0	20.6–23.4

^a All strains were grown on complex BAP indicator (TYE-XP) agar with added ampicillin for pBW20 and pTHR32, kanamycin for pMW1, chloramphenicol for pJP72 and pRPG1, and tetracycline for pJP86, at 100, 50, 100, and 10 µg/ml, respectively.

^b Strain BW2091 [Δ (*phoM*)93 *phoR*68] was transformed with all plasmids except pTHR32, for which the analogous strain BW2867 [Δ (*phoM*)93 *phoR*68] was used.

^c The approximate incubation time at 37°C is given.

^d Six colonies of each clonal type were assayed as described previously (19). Specific activity is expressed in units per an optical density at 420 nm. The arithmetic mean is given.

wild-type *phoM* chromosomal region. In other words, whereas variability was dominant when the wild-type *phoM* operon was on a multicopy plasmid, constitutivity was dominant when the *pho-510* allelic form of the *phoM* region was on the same multicopy plasmid.

Although these data could indicate that whichever allele is in a higher copy number is dominant, there exists a caveat to this interpretation. P1 transductants of strains such as BW2091, as well as closely related ones in the XPh1a lineage (19, 20), which contain a wild-type *phoM* chromosomal region, do not show a normal pattern of variation. Instead, XPh1a *phoR* mutants that received the wild-type *phoM* region in P1 crosses generally appear to be constitutive; such transductants yield only occasional negative variants that are difficult to purify (unpublished data). As a consequence, even a small effect of the *pho-510 phoM* plasmid on the apparent frequency of variation in XPh1a *phoR phoM*(wt) mutants could totally obscure variation. This could be due to an effect of plasmid copy number on variation. An effect of plasmid copy number is also indicated by the observation that XPh1a *phoR* mutants containing a multicopy *phoM*(wt) plasmid display the Bap clonal variation phenotype, as described in this paper, even though XPh1a *phoR* transductants with a chromosomal wild-type *phoM* operon fail to display a normal pattern of variation (data not shown).

DISCUSSION

The wild-type *phoM* operon was molecularly cloned. When the plasmids were used to complement Bap-negative Δ *phoM phoR* mutants, the transformants showed an unstable Bap phenotype. Further studies showed that the complemented mutants alternately expressed a Bap-constitutive and -negative phenotype. The alternation was bidirectional and, like *phoM*-dependent BAP synthesis (25), acted at the transcriptional level for the *phoA* gene. Also, the *phoM* plasmids had no effect on BAP synthesis in *phoR*⁺ bacteria.

It was suspected that the metastable Bap phenotype could be due to an instability in the *phoM* plasmids. However, no evidence for a DNA change was detected either in the plasmid or chromosome of the transformants. Also, the alternation was *recA* independent. Since a small DNA change in the plasmid could have gone undetected, plasmid DNAs were isolated from both variant types and examined in transformations of Bap-negative Δ *phoM phoR* mutants. No association was found between the proportion of transformant clonal types and the phenotypes of the variants from which the plasmid DNAs were isolated. We concluded that the alternating Bap phenotype is unlikely to be due to a DNA change in or near the *phoM* operon. A change in the chromosome could have gone undetected. This is because multiple steps were required in the curing and retransformation tests that were done. Such manipulations could have led to mixed cell populations that no longer reflected the original clonal type.

While we were studying the Bap clonal variation phenotype with *phoM*(wt) plasmids, several others reported the molecular cloning of the *phoM* operon (2, 9, 10, 15, 22). Except for one study with the plasmid pRB1 (2), no mention was made of an unstable Bap phenotype. The apparent instability with pRB1 was not investigated in detail. We showed that the *phoM* plasmid pRB1, like its predecessor pRPG1, showed the variable Bap phenotype. The metastable behavior for this plasmid could explain some seemingly contradictory data regarding the location of the *phoM* gene

on the plasmid pRB1 (2). The other *phoM* plasmids that we had been able to examine conferred a Bap-constitutive (stable) phenotype in Δ *phoM phoR* mutants.

An explanation was sought to explain why certain *phoM* plasmids gave the metastable Bap phenotype and others gave the constitutive (stable) phenotype. A physical comparison of the various *phoM* plasmids revealed that the same chromosomal region was cloned. However, we had noticed several years earlier that some *phoR* mutants made by P1 transduction also showed an unstable Bap phenotype (17; unpublished data). A further examination of the seemingly unstable *phoR* transductants revealed that they, like the *phoM*(wt) plasmid transformants, also displayed a bidirectional clonal variation phenotype with respect to Bap synthesis (17). That variability was also *recA* independent and affected *phoA* transcription (17, 19). Furthermore, whether *phoR* mutants showed the metastable behavior or a constitutive (stable) Bap phenotype depended upon a genetic difference among strains in the *phoM* region. The *phoR* mutants with the wild-type *phoM* operon show the Bap clonal variation phenotype, and mutants with the *pho-510* mutant form of the *phoM* region synthesize BAP constitutively (20). We examined the strains from which the DNA was derived to make the various *phoM* plasmids. This revealed that plasmids made from bacteria with the wild-type *phoM* operon displayed the metastable Bap phenotype, whereas plasmids made from bacteria with the *pho-510* allelic form of the *phoM* operon led to constitutivity. We concluded that the different phenotypes shown by *phoM* plasmids were due to the DNA source used to clone the *phoM* region. This was further substantiated by the isolation of Var⁻ derivatives of the plasmid pMW1(Var⁺) in *recA*⁺ *pho-510* mutants.

Bap clonal variation in *phoM*(wt) plasmid transformants is, in general, similar to the variation in *phoR* mutants that are otherwise wild type and have the chromosomal *phoM*(wt) region, as described previously (19, 20). Two differences were seen, however. First, the Bap-negative Δ *phoM phoR* mutant BW2091 (23), which has the Δ (*trpR-phoM-dye-thr-chlG*)93 deletion, does not show a normal Bap clonal variation phenotype when a chromosomal copy of the *phoM*(wt) operon is crossed into it with P1 by selecting Thr⁺ transductants. Although Bap-negative variants are seen, it is difficult to purify them. [The presence of the *phoM*(wt) allele could be verified in such transductants by the appropriate backcrosses to a *phoM* tester strain (20; unpublished data).] Since several related strains in the XPh1a lineage (19, 20) also tend not to yield Bap-negative variants frequently, another mutation(s) in the XPh1a background could affect variability. Nevertheless, variation was seen when multicopy *phoM*(wt) plasmids were transformed into the Bap-negative Δ *phoM phoR* mutant BW2091 of the XPh1a lineage (20). These data suggest that multiple plasmid copies of the *phoM*(wt) region may affect variability.

Another difference in *phoM*(wt) plasmid transformants concerns the physiological control of variation. In other experiments, Bap clonal variation was dramatically affected by the growth medium, especially by the presence of glucose (19, 20). No effect of glucose was seen in the transformants, however. Instead, the metastable Bap phenotype was seen on all media tested. The reduced variability in XPh1a-derived *phoM*(wt) *phoR* transductants and the apparent lack of physiological control over variation in multicopy *phoM*(wt) plasmid transformants in the same genetic background could be related. A molecular understanding of these effects must await future studies.

Complementation tests with multicopy plasmids carrying the *phoM*(wt) or *pho-510 phoM* operon showed that both alleles were dominant over Bap-negative *phoM* operon point mutations. However, when tested against each other, it was unclear whether the wild-type *phoM* or *pho-510 phoM* operon allele was dominant. The dominant character instead seemed to associate with the allele in multicopy. A failure to detect variability in *pho-510 phoM* plasmid transformants of a *phoM*(wt) *phoR* mutant could be due to the reduced variability already seen in the isogenic *phoM*(wt) transductants lacking a plasmid, as mentioned above. Complementation tests were done, therefore, between the *phoM*(wt) and *pho-510* alleles when both were in single copy. In this case, a λ *phoM*(wt) lysogen of a *recA phoR* mutant containing a chromosomal copy of the *pho-510 phoM* operon showed the Bap clonal variation phenotype (Wilmes and Wanner, unpublished). We conclude that the wild-type *phoM*(wt) allele is dominant, at least when each allele is in single copy. However, a more definitive complementation study will require the construction of well-defined lesions in a wild-type *phoM* background. The only available *phoM* operon point mutations were made in its *pho-510* mutant form (20, 25).

Since variability appears to be dominant in heterozygous *phoM*(wt)/*pho-510* mutants, the *pho-510* mutation probably leads to the loss of a function. It therefore should be possible to identify insertion mutations in the *phoM* operon which show a Bap-constitutive phenotype in *phoR* mutants. Such an insertion upstream of the *phoM*(ORF3) gene would have to be nonpolar to allow expression of the *phoM* gene itself. Although many transposon-induced mutations in the *phoM* operon are Bap negative, one that maps upstream of the *phoM* gene leads to a Bap-constitutive phenotype (Wilmes and Wanner, unpublished). Accordingly, such an insertion is a candidate for a mutation in ORF1 or ORF2 of the *phoM* operon. Further studies of the *phoM*(wt) operon are in progress.

The *phoM* gene was discovered by its effect on BAP synthesis in *phoR* mutants (25). In *phoR*⁺ bacteria, no *phoM* operon mutation appears to affect *phoA* gene expression (22, 23, 25; Wilmes and Wanner, unpublished); even deletions that remove the entire *phoM* operon are without detectable effect on normal BAP regulation (23). The lack of an effect of the *phoM* operon in *phoR*⁺ cells and the unusual Bap phenotype in *phoR* mutants suggest that the *phoM* operon may normally have another role which is unknown. This role could be related to the control of BAP synthesis that is seen in *phoR* mutants with the wild-type *phoM* operon.

The sequence similarities at the protein level for the *phoM* operon genes with other regulatory genes (1, 12) suggest that the ORF2 and ORF3(*phoM*) proteins may have a regulatory function. It is reasonable to speculate that the normal role of the *phoM* operon (in *phoR*⁺ cells) is not to regulate *phoBR*-dependent promoters. Rather, its role could be to regulate a different set of promoters in response to an unknown environmental signal. Since glucose affects Bap clonal variation in *phoR* mutants (17, 19, 20; unpublished data), the *phoM* operon may normally play a role in catabolite control of gene expression in bacteria.

We propose that the Bap clonal variation phenotype in *phoR* mutants reflects an underlying gene regulatory mechanism, which may have a fundamental role in bacteria that is not specifically related to its effect on the phosphate regulon in *phoR* mutants. On the basis of the physiological control of the Bap clonal variation phenotype, we suggest that regulatory genes in the *phoM* operon may normally regulate an

unknown set of genes in response to environmental levels of glucose or another catabolite. The *phoM*-dependent control over *phoBR*-dependent promoters of the phosphate regulon in *phoR* mutants (22, 25), in which the phosphate sensory protein is abolished, may reveal "crosstalk" (12) among different environmentally responsive gene systems.

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