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Construction of a Tightly Regulated Plasmid Vector for *Streptococcus pneumoniae:* Controlled Expression of the Green Fluorescent Protein

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We have constructed a regulated plasmid vector for *Streptococcus pneumoniae,* based on the streptococcal broad-host-range replicon pLS1. As a reporter gene, we subcloned the *gfp* gene from *Aequorea victoria,* encoding the green fluorescent protein. This gene was placed under the control of the inducible P_M promoter of the *S. pneumoniae malMP* operon which, in turn, is regulated by the product of the pneumococcal *malR* gene. Binding of MalR protein to the P_M promoter is inactivated by growing the cells in maltose-containing media. Highly regulated gene expression was achieved by cloning in the same plasmid the P_M-*gfp* cassette and the *malR* gene, thus providing the MalR regulator *in cis.* Pneumococcal cells harboring this vector gave a linear response of GFP synthesis in a maltose-dependent mode without detectable background levels in the absence of the inducer.

Key Words: pneumococcal *malR* gene; plasmid pLS1; regulated promoters.

Streptococcus pneumoniae (pneumococcus) is an important pathogenic gram-positive bacterium causing a variety of illnesses, such as pneumonia, otitis, and septicemia. Pneumococcal-provoked diseases constitute an important problem because of the growing number of strains developing resistance to antibiotics, specially β-lactams (Baquero, 1996). Even though pneumococci are naturally transformable bacteria, only a few plasmid vectors are available to manipulate them, most of them isolated from heterologous hosts (Muñoz *et al.,* 1999, and references therein). Indigenous cryptic pneumococcal plasmids have been recently characterized and shown to belong to the pC194/pUB110 plasmids replicating by the rolling circle mechanism (Schuster *et al.,* 1998). A new shuttle vector between *S. pneumoniae* and *Escherichia coli,* based on an indigenous cryptic pneumococcal plasmid, has been recently constructed (Muñoz *et al.,* 1999).

Cloning of pneumococcal genes (Stassi *et al.,* 1982) was feasible early by employment of plasmid pLS1, a derivative of the *S. agalactiae* pMV158 natural replicon (Burdett, 1980). Although the entire pneumococcal genome has been sequenced, still only a few vectors, mostly based on the pMV158 replicon, have been constructed. They have been used either as vehicles to clone pneumococcal genes (Lacks *et al.,* 1986) or as promoter probe plasmids (Díaz and García, 1990). The promiscuity of the pMV158 replicon has allowed its use as a broad-host-range vector to analyze heterologous expression of cloned pneumococcal genes (Espinosa *et al.,* 1984; López *et al.,* 1987).

During the past few years, we have been interested in the study of the utilization of maltosaccharides by *S. pneumoniae* (Stassi *et al.,* 1982; Puyet and Espinosa, 1993). Genes involved in this process are organized as a regulon, which is composed of three operons (see Fig. 1A). Two of them are involved in maltosaccharide uptake (*malXCD*) and its utilization (*malMP*), and the third (*malAR*) is involved in regulation (Puyet and Espinosa, 1993; Puyet *et al.,* 1993). The two former operons are transcribed from two divergent promoters, termed P_M (for the *malMP* operon) and P_X (for the *malXCD* operon) which are negatively regu-

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lated by the product of gene *malR* (Nieto *et al.*, 1997). Protein MalR belongs to the LacI–GalR family of transcriptional repressors (Puyet *et al.*, 1993) and binds specifically to two operator sequences located in the intergenic region between operons *malXCD* and *malMP* (Nieto *et al.*, 1997). The binding of MalR to its DNA target is reversed by the addition of maltose, pointing to inactivation of the repressor by the sugar (Nieto *et al.*, 1997). Based on this regulatory mechanism, in the present work we have developed a pLS1-based plasmid, which harbors the *malR* gene *in cis* and which has proved to be a useful regulated expression vector for *S. pneumoniae*. We have employed the *gfp* gene from *Aequorea victoria* as a reporter, encoding the green fluorescent protein (GFP),[2] placed under the control of one of the promoters regulated by MalR.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Most of the constructions were done in *E. coli* JM109 (Sambrook *et al.*, 1989). The final receptor of the constructions was *S. pneumoniae* R61 (wild type; Lacks, 1968). These latter bacteria were also employed for protein expression and measurement of GFP activity. In addition to the plasmids detailed below (see Results), we used plasmids pBR322 (Bolivar *et al.*, 1977) and pJDC9 (a pMB9 derivative; Chen and Morrison, 1987). This latter plasmid carries the *lacZ′* gene (allowing for blue/white colonies selection) and strong transcription termination sequences flanking the multiple cloning site (MCS), thus facilitating the cloning of streptococcal DNA fragments in *E. coli* (Chen and Morrison, 1987). In addition, we employed plasmid pGreen TIR (Miller and Lindow, 1997), which is a derivative of pUC1813 into which a mutated *gfp* gene (a double F64L S65T amino acid change) has been cloned. This plasmid also contains an improved translation initiation region (TIR), containing an optimized Shine–Dalgarno sequence and spacer region, and a translational enhancer from gene 10 of phage T7. The resulting *gfp* gene cassette is suitable for prokaryotes, and the total fluorescence is enhanced (Miller and Lindow, 1997). Other plasmids used were pAPM22, lacking the *gfp* gene (Puyet *et al.*, 1993), and pLS1GFP, which harbors *gfp* under the control of promoter P_M and lacks *malR* (Fernández de Palencia *et al.*, 2000).

E. coli cells were grown in TY medium (Sambrook *et al.*, 1989) with selection for erythromicyn resistance (Erm^R; 500 μg/ml) or ampicillin resistance (Ap^R; 100 μg/ml) for cells harboring pJDC9 or pGreenTIR, respectively. *S. pneumoniae* cells were grown in AGCH medium (Lacks, 1968) supplemented with sucrose, maltose, or both sugars at the final concentration of 0.8%. Selection was applied for Erm^R (1 μg/ml). All cultures were grown at 37°C.

Plasmid DNA Preparation and DNA Manipulations

Pneumococcal cells were grown and transformed with plasmid DNA as previously described (Stassi *et al.*, 1982; Lacks *et al.*, 1986). Preparation of *E. coli* competent cells to electroporate with plasmid DNA was done as described (Sambrook *et al.*, 1989). Extraction and purification of plasmid DNAs from *E. coli* were performed by use of the Qiagen kit (Qiagen, U.S.A.). Purified plasmid DNAs from *S. pneumoniae* were prepared as described (del Solar *et al.*, 1987). Restriction endonucleases, the Klenow fragment of DNA polymerase I (PolIK), and DNA ligase were purchased from New England Biolabs or Boehringer Mannheim and were used as specified by the suppliers. Restriction fragments employed for cloning experiments were purified from agarose gels by phenol extraction (Sambrook *et al.*, 1989). Determination of the nucleotide sequence of the desired DNA fragments was performed on an automated sequencer (ABI 373A) and the dye–dideoxynucleotide termination procedure.

To amplify the pneumococcal region contain-

[2] Abbreviations used: Ap, ampicillin; Erm, erythromycin; GFP, green fluorescent protein; *gfp*, gene encoding GFP; MCS, multiple cloning site; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PolIK, Klenow fragment of DNA polymerase I; TIR, translation initiation region.

ing promoters P_X and P_M by polymerase chain reaction (PCR), plasmid pLS70 was used as a template. This plasmid contains a *S. pneumoniae* region encompassing both promoters (Stassi *et al.*, 1982). The oligonucleotides used included, respectively, recognition sequences for the restriction enzymes *Eco*RI and *Bam*HI (underlined): mal3 (promoter P_X), 5′-GCAGAATTCAAGTTTTATTGATAAGGAAAC-3′; mal4 (promoter P_M), 5′-CGCGGATCCATCTCTAGAGTATTTTGCAGACGCAAACG-3′.

Preparation of Total Proteins from S. pneumoniae and SDS Gels

Pneumococcal cultures harboring plasmids were grown to an optical density (OD_{650}) of 0.6 (equivalent to 5×10^8 colony-forming units/ml) in sucrose-containing medium (Lacks, 1968). Cells were washed and diluted (1/10) in medium containing sucrose or maltose (final concentration, 0.8%). Cultures were grown to the same OD and harvested by centrifugation. Total protein extracts were prepared as described (Espinosa *et al.*, 1984) and were separated by SDS–polyacrylamide (12.5%) gel electrophoresis (PAGE). As molecular weight standards, the Mark 12 protein mixture (Novex, U.S.A.) was used. The band corresponding to GFP protein was quantified by use of the Gel Doc-2000 equipment and Quantity One software (Bio-Rad Laboratories).

Western Blot Analysis

Pneumococcal cells harboring plasmids were grown in medium containing sucrose, maltose, or both sugars. Total protein extracts (25 μg) were separated by SDS–PAGE as above, using as molecular weight markers the Kaleidoscope prestained protein mixture (Bio-Rad). Proteins were electrotransferred to nitrocellulose membranes (Schleicher & Schuell) in buffer A (25 mM Tris–HCl, 192 mM glycine, pH 8.3, 0.1% SDS, 20% methanol), 300 mA, 4°C, 60 min, and treated essentially as reported (Bravo and Salas, 1997). Polyclonal GFP antibodies (Clontech, U.S.A.; diluted 1:3000) were bound to the membrane for 3 h at room temperature in TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 0.2% skimmed milk. The membrane was rinsed four times with the same buffer, and the secondary antibody (anti-rabbit IgG, diluted 1:30,000; Sigma Immunochemicals) was added. Incubation proceeded for 1 h, and the membrane was rinsed with TBS containing 0.2% Tween 20. Immunodetection was performed using an Immun–Star kit (Bio-Rad), as described by the manufacturers. For autoradiography, the membrane was exposed to Kodak X-Omat film for 1–3 min. Quantitative estimates of the intensity of the bands were done by densitometric scannings of the autoradiograms, correcting for the protein concentration loaded for each sample.

Measurement of GFP Activity

Pneumococcal cells harboring plasmids were grown in sugar-containing media to an OD_{650} of 0.7 (about 6×10^8 cfu/ml) as above, with selective pressure. Cells (1 ml from each culture) were sedimented by centrifugation and suspended in the same volume of PBS buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl), pH 7.2. Aliquots (200 μl) were used to measure the fluorescence on a LS–50B spectrophotometer (Perkin–Elmer) by excitation at a wavelength of 488 nm and detection of emission at 510 nm. As a control to determine the background fluorescence, cells harboring plasmid pAPM22 (lacking the *gfp* gene) were used.

RESULTS AND DISCUSSION

Construction of Plasmid pJDC9GFP

A schematic representation of the various cloning steps for constructing the intermediate plasmid pJDC9GFP given in Fig. 1. The DNA region containing the operators/promoters P_X and P_M, the targets of the repressor MalR (Fig. 1A), was amplified by PCR. Two restriction sites for *Eco*RI and for *Bam*HI flanked the amplified DNA (0.5 kb). This region lacks the

TIR for *malX* and *malM* genes. Plasmid pBR322 was doubly digested with *Eco*RI–*Bam*HI (Fig. 1B). Plasmid and PCR-synthesized DNA products were ligated together and used to transform *E. coli* JM109, applying selection for Ap^R. Twenty tetracycline-sensitive clones were selected and analyzed for plasmids of the expected size. Eight of the clones contained a plasmid, termed pCL1, which was further characterized by restriction mapping. To clone the *gfp* gene into pCL1, DNA from plasmid pGreen TIR was digested with *Eco*RI and treated with PolIK, and the 0.75-kb DNA fragment containing the *gfp* gene with its own TIR signals was purified. Plasmid pCL1 DNA was digested with *Bam*HI and treated with PolIK. Both DNAs were ligated and used to transform *E. coli* JM109. Clones harboring a functional *gfp* gene were selected by checking the fluorescence of the Ap^R colonies under UV light. Several clones were checked for their DNA content, and the resulting plasmid was termed pCL1GFP. The fluorescence of *E. coli*/pCL1GFP showed substantial variations among different clones (not shown). We attributed these variations to the lack of *gfp* transcription terminators in pCL1GFP. For this reason, we decided to clone the P_X–P_M promoters–*gfp* gene cassette (contained within an *Eco*RI–*Sal*I DNA fragment) into plasmid pJDC9. DNA from this plasmid was doubly digested with *Eco*RI–*Sal*I (both within the MCS). Selection was applied for Erm^R, and white colonies exhibiting fluorescence under UV light were picked. Eight clones were randomly chosen and checked for plasmids of the expected size, and one of these clones was further selected to determine the nucleotide sequence of the pneumococcal insert. The resultant recombinant plasmid was termed pJDC9GFP (Fig. 1B).

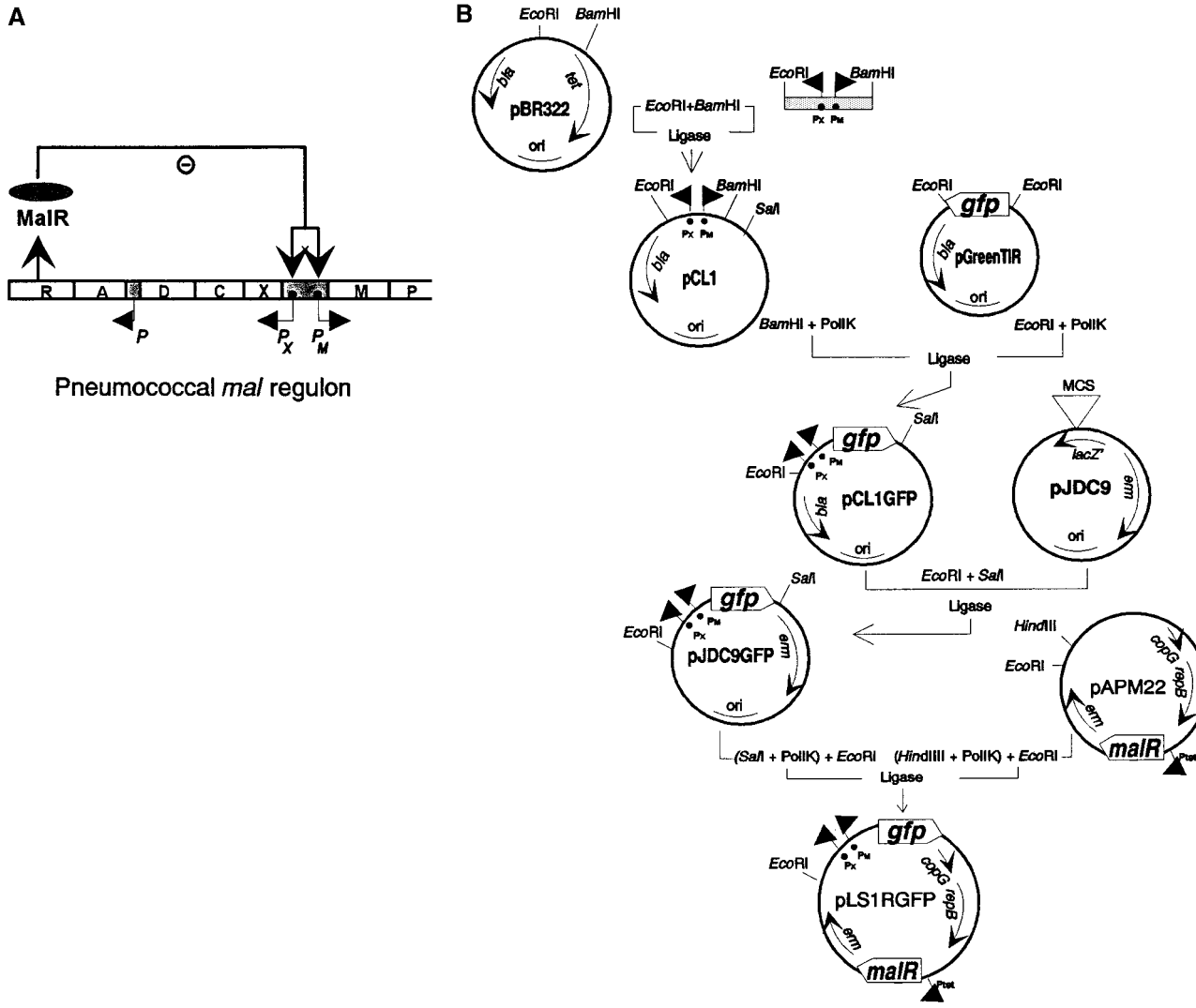
Construction of pLS1RGFP, A Regulated Plasmid Vector for S. pneumoniae

To construct a regulated plasmid vector we placed the promoter P_M–*gfp* gene cassette (present in pJDC9GFP) into a pLS1-based replicon. To increase the intracellular amount of MalR, thus ensuring high levels of repression, the *malR* gene was cloned into the same plasmid. To this end, we made use of the previously constructed plasmid pAPM22 (Fig. 1B), which has the pLS1 replicon and contains the *malR* gene cloned under the control of the promoter for the *tet* gene of pLS1 (Puyet *et al.,* 1993). DNA from pAPM22 was digested with *Hin*dIII, treated with PolIK, and digested with *Eco*RI, and the larger fragment was purified. Plasmid pJDC9GFP DNA was digested with *Sal*I, treated with PolIK, and then digested with *Eco*RI. The 1.5-kb DNA fragment (containing *gfp*) was purified and ligated to the pAPM22 DNA fragment. The ligation mixture was used to transform *S. pneumoniae,* and transformants were selected for Erm^R. Due to the high intrinsic fluorescence of the pneumococcal medium, direct selection for Erm^R and fluorescent colonies was not feasible. Plasmid DNAs from several clones having an appropriate size were analyzed by restriction mapping, and one of them was selected. The resulting plasmid was termed pLS1RGFP (Fig. 1B), and the integrity of the P_M–*gfp* gene cassette was checked by determination of its entire nucleotide sequence.

Synthesis of GFP in S. pneumoniae/pLS1RGFP

To determine whether synthesis of GFP was regulated in pneumococcal cells harboring pLS1RGFP, cultures were grown in medium containing sucrose or maltose (the inducer of the *mal* regulon). As controls, we used plasmids

FIG. 1. Construction of pLS1RGFP. (A) Schematic representation of the pneumococcal *mal* regulon showing the organization of the different genes (letters within rectangles). Promoters directing transcription of the *malAR, malXCD,* and *malMP* operons are indicated by arrowheads. The negative regulation (−) of promoters P_M and P_X (shadowed) by MalR (arrows) is indicated. (B) Construction of pLS1RGFP. Plasmid-encoded genes are indicated with thin lines, the arrowheads pointing in the direction of transcription. The two relevant genes, *gfp* and *malR,* are indicated as empty boxes, the narrower ends pointing in the direction of transcription.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tet
pBR322
ori
EcoRI+BamHI
Ligase
Px Pm
SalI
pCL1
BamHI + PolIK
gfp
pGreenTIR
EcoRI + PolIK
pCL1GFP
MCS
lacZ'
erm
pJDC9
pJDC9GFP
HindIII
copG
repB
pAPM22
malR
Ptet
(SalI + PolIK) + EcoRI
(HindIII + PolIK) + EcoRI
pLS1RGFP

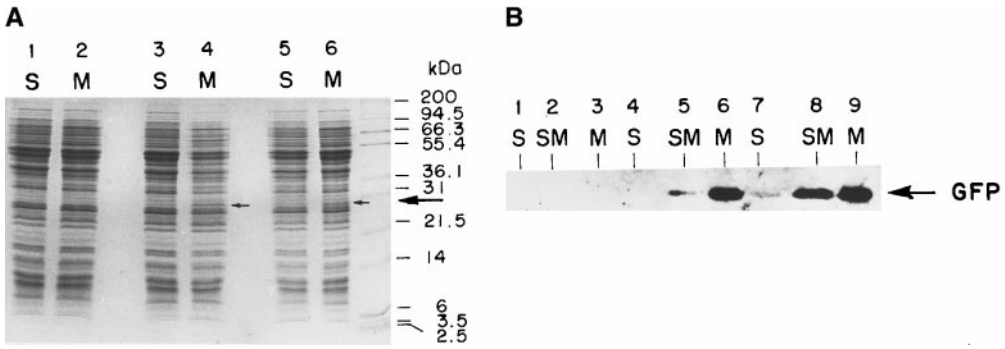

FIG. 2. Relative synthesis of GFP in pneumococcal cells harboring plasmids. (A) Extracts of cells of *S. pneumoniae*/pAPM22 (lanes 1 and 2), pLS1GFP (lanes 3 and 4), and pLS1RGFP (lanes 5 and 6) grown in sucrose-containing (S) or maltose-containing (M) medium were fractionated by SDS–PAGE. Samples contained 25 μg of proteins. Arrows point to the position corresponding to GFP. The lane on the right shows the migration of the proteins of the molecular weight standards, with their sizes indicated on the right. (B) Immunodetection of GFP by Western blotting of total protein extracts from pneumococcal cultures harboring plasmids (as in A), grown in medium supplemented with sucrose (S), sucrose plus maltose (SM), or maltose (M).

pAPM22 (lacking *gfp*) and pLS1GFP (carrying *gfp* but not *malR*). Wild-type pneumococcal cells harboring the latter plasmid have a single chromosomal copy of the gene encoding the repressor *malR* but multiple copies of the promoter/operator region of *malM*, a genetic configuration that should lead to a poor regulation of *gfp* expression. Total protein extracts were prepared, separated by electrophoresis on SDS–PAGE, and stained. The results showed that induced cells harboring pLS1RGFP synthesized a protein band of the expected size of GFP, with a relative electrophoretic mobility of M_r of about 28,000 and amounting to about 1% of the total protein content (Fig. 2A). This band was also visible in cells harboring pLS1GFP, but it was absent in *S. pneumoniae*/pAPM22. To test whether this band corresponded to GFP, Western blot analysis was performed. In this case, cells were grown in media containing sucrose, maltose, or a mixture of both sugars (to achieve a partial repression of the system). Proteins were separated by SDS–PAGE as above, synthesis of GFP was tested by immunodetection, using anti-GFP antibodies (Fig. 2B), and the bands were quantified by densitometric scanning of the autoradiograms (Table 1). In the case of pLS1GFP, detectable amounts of GFP were synthesized even under conditions of repression (sucrose), although GFP synthesis was increased about three- or sevenfold by growing the cells in media containing maltose plus sucrose or only maltose, respectively. These findings indicate that the product of a single chromosomal copy of *malR* exerts a weak regulation on the multiple copies of the plasmid-borne P_M promoter. However, in the case of *S. pneumoniae*/pLS1RGFP a clear regulation of GFP synthesis was observed, since no signal was observable when the cells were grown in medium containing sucrose (Fig. 2B). No signal was found in cells harboring the control plasmid pAPM22 under any condition.

TABLE 1

Relative Quantification of Immunodetection of GFP Synthesized by *S. pneumoniae* Cells Harboring Plasmids Grown in Sugar-Supplemented Media

	Sugar		
Plasmid	Sucrose	Sucrose + maltose	Maltose
pAPM22	ND[a]	ND	ND
pLS1GFP	1.12	3.5	7.2
pLS1RGFP	ND	1.0[b]	10.5

[a] ND, undetectable.

[b] This relative value was given to the lowest value detectable.

TABLE 2

Intensity of Fluorescence in Pneumococcal Cultures Harboring Plasmids

Plasmid	Sugar		
	Sucrose	Sucrose + maltose	Maltose
pAPM22	5.5 ± 0.5	5.4 ± 0.15	4.9 ± 0.12
pLS1GFP	10.5 ± 0.53	19.9 ± 1.8	54.3 ± 2.2
pLS1RGFP	5.3 ± 0.87	16.2 ± 0.52	64.5 ± 1.4

Densitometric scanning of the autoradiograms (Table 1) indicated regulation of GFP synthesis in *S. pneumoniae*/pLS1RGFP, since the relative value of the GFP band was 10 times higher in maltose-grown cells than in those grown in maltose plus sucrose, and no detectable signal could be measured for sucrose-grown cells. We conclude that plasmid pLS1RGFP constitutes an excellent vector for *S. pneumoniae* when tightly controlled gene expression is desired.

Detection of gfp Expression in S. pneumoniae by Determination of Fluorescence Levels

Measurement of GFP activity requires a post-translational oxidation of the protein, and full activity is only achieved at pH around 7.0 (reviewed in Sullivan and Kay, 1999). These features are reversible, but for microaerophile acidifying bacteria (like *S. pneumoniae*), the cells must be exposed to aerobic and neutral conditions and fluorescent compounds in the culture media must be removed. These inconveniences have hindered the use of GFP as a tool for pneumococci and, in fact, we have been unable to detect fluorescence in the pneumococcal colonies. However, fluorescence could be routinely measured in exponentially growing cultures which were centrifuged, washed, and resuspended in PBS buffer.

To determine the levels of *gfp* expression in *S. pneumoniae* cells harboring pLS1RGFP, pLS1GFP, or pAPM22, cultures (in triplicate) were grown in medium containing sucrose, maltose, or both sugars. After suspension in PBS buffer, emission of fluorescence at 510 nm was measured. The results (Table 2) showed that *S. pneumoniae*/pLS1RGFP cells grown in sucrose gave background values (those obtained in the case of pAPM22). When the cells were grown in maltose-containing medium, a 10-fold increase in fluorescence was observed, thus confirming the approximate quantifications derived from the Western blot analyses.

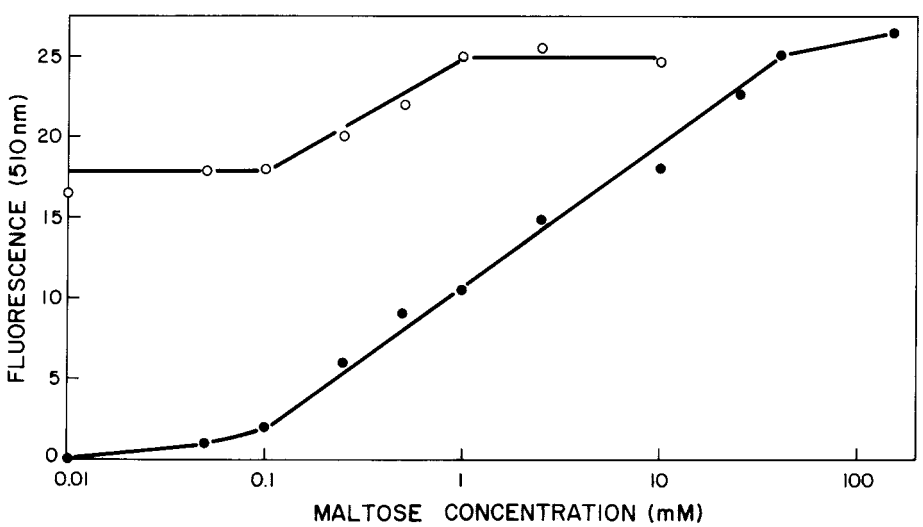

FIG. 3. Detection of fluorescence in cultures of *S. pneumoniae*/pLS1GFP (○) or *S. pneumoniae*/pLS1RGFP (●) as a response to increasing amounts of maltose in the medium.

Linearity of GFP Synthesis in S. pneumoniae/pLS1RGFP as a Response to Maltose Concentration

To test whether *S. pneumoniae*/pLS1RGFP responded to increasing concentrations of the inducer, cells were grown in sucrose-containing medium to an OD_{650} of 0.3. Then, cells were centrifuged, washed, and resuspended in the same medium containing increasing amounts of maltose. Incubation was resumed for one doubling time, and at this time fluorescence of the cultures were assayed, correcting for the fluorescence of cells harboring pAPM22. As a control, cells harboring pLS1GFP were also tested. The results (Fig. 3) showed that the levels of GFP increased linearly with the concentration of maltose in a wide range of this sugar (between 0.1 and 25 mM), whereas in the case of pLS1GFP, again a weak regulation was observed. These findings demonstrate that a fine tuning for regulation of gene expression is achieved by the employment of pLS1RGFP. We believe that this plasmid will constitute a valuable tool for the manipulation of gene expression in *S. pneumoniae.*

CONCLUSIONS

1. Expression of the prokaryotic-optimized *gfp* gene can be detected and quantified in *S. pneumoniae.*

2. Synthesis of GFP depended upon the growth of pneumococcal cells in maltose-containing medium.

3. Plasmid pLS1RGFP constitutes a tightly regulated pneumococcal vector.

4. The amount of GFP synthesized in cells harboring pLS1RGFP increased linearly with the concentration of maltose employed.

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