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The Expression of the *Photinus pyralis* Luciferase Gene in *Staphylococcus aureus* Cowan I Allows the Development of a Live Amplifiable Tool for Immunodetection

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We expressed the *luc* gene, encoding luciferase from *Photinus pyralis*, in *Staphylococcus aureus* Cowan I downstream of the plasmid-borne promoter for protein A. Constitutive luciferase synthesis did not impair the growth rate of the host nor did it affect the stability of the plasmid. Light production started immediately after addition of luciferin. The kinetic profile is of the glowing rather than the peak type. Because *S. aureus* Cowan I produces large quantities of protein A, of which a substantial part becomes covalently attached to rigid cell walls, the bacterial cells could be specifically immobilized on a substrate to which immunoglobulin G molecules were adsorbed either directly or as secondary antibodies. Light production from these cells can be used as a reporter tool for the detection of antigen-antibody complexes. Fourfold amplifications of the emitted signals were obtained by in situ incubation of the bound cells in bacterial growth medium.

One of the more striking features of most clinical isolates of *Staphylococcus aureus* is the presence in the cell walls of large amounts of protein A, a protein which is able to bind certain isotypes of immunoglobulin G (IgG) molecules (9). The Ig binding capacity resides within the amino-terminal part of protein A, which consists of five homologous domains and extends into the growth medium. The carboxy-terminal parts of protein A molecules are bound covalently to the peptidoglycan layer of the cell wall (18). Cowan's serotype I, commonly designated Cowan I, is an isolate of *S. aureus* (ATCC 12598), which is known as a high-level producer of protein A (1).

The luciferase of *Photinus pyralis*, a light-producing beetle, has long been used for the detection and titration of ATP (5). The *luc* gene, encoding a single polypeptide, has been cloned in *Escherichia coli* (7) and has proven to be a valuable tool in the study of promoter activity in various host organisms and cell types of both prokaryotic (15) and eukaryotic (6) origins. The gene has also proven to be a suitable marker in transgenic plants (14) and animals (8). The Luc protein does not need auxiliary modifications to achieve optimal activity (7) and exhibits a very high quantum yield (17).

We subcloned the *luc* gene downstream from the constitutive promoter for *S. aureus* protein A (*psa*) on a high-copy-number plasmid. We report here the functional expression of the Luc protein in *S. aureus* Cowan I. This strain showed a continuous, stable emission of light over at least 30 min. This recombinant strain could be used as a live reporter tool for immunodetection. A system for specific amplification of emitted light signals is described.

MATERIALS AND METHODS

Bacterial strains and general plasmid DNA procedures. All plasmid constructions were performed with *E. coli* MC1061 [*araD139* Δ(*araABC-leu*)*7697* Δ(*lac*)*X74 galU galK rpsL thi-1 hsdR2 mcrA mcrB1*] (4) because of its high efficiency of transformation. The *S. aureus* strain RN4220 (11) was used as a primary recipient for *E. coli* DNA because of its weak restriction barrier (1). Binding studies using *S. aureus* Cowan I (ATCC 12598) were performed. Restriction enzymes (Boehringer, Mannheim, Germany, or Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used as recommended by the manufacturers. Standard techniques for the isolation of plasmid DNA from *E. coli* (3, 10) were used. Restriction fragments of DNA were prepared by the GeneClean method (23). Plasmid purification for subsequent electroporation was performed by the same technique.

S. aureus plasmid DNA was prepared from 20 ml of overnight cultures, grown at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) under antibiotic selection (chloramphenicol at 5 μg ml^{-1}). The cells were collected and washed three times with 1 volume of a 20 mM Tris-HCl (pH 7.5)–50 mM EDTA solution. The cells were then resuspended in 5 ml of the same solution, to which 0.3 mg of lysostaphin (Sigma, St. Louis, Mo.) was added. After incubation at 37°C for 2 h, 5 ml of a solution of 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, and 0.2% Triton X-100 was added. The suspension was centrifuged for 30 min at 15,000 rpm in a Sorvall SS34 rotor to remove chromosomal DNA, and the supernatant was extracted three times with phenol. Trace amounts of phenol were removed by extraction of the water phase with 1 volume of diethyl ether, and plasmid DNA was precipitated from the aqueous phase by addition of 0.7 volume of 2-propanol.

Transformation of *S. aureus*. *S. aureus* was transformed by electroporation, essentially according to the method described for *Staphylococcus epidermidis* (2). We observed an optimal level of efficiency of 10^5 transformed cells per μg of plasmid DNA when cells were harvested at an optical density at 578 nm of 0.700. Samples were electroporated in a 1-mm-gap-size cuvette with an electroporator (Invitrogen, NV, Leek, The Netherlands) with a 50-μF capacitor, a field strength of 1 kV, and a 100-Ω resistance. Immediately after being pulsed, the samples were diluted with 1 ml of LB medium and incubated for 30 min at 37°C prior to plating on selective agar.

Construction of plasmids. All steps in the construction of the *luc* expression plasmid pPSALUC2 are depicted in Fig. 1 and 2. A 0.6-kb *Bam*HI-*Eco*RI fragment containing the 5′ end of the *luc* gene was isolated from plasmid pSVOAΔL5′ (6) and ligated in the *Bam*HI-*Eco*RI-opened plasmid pMa58 (19) to give pMaLUC1. Site-directed mutagenesis (12) was performed on this plasmid with a 29-mer oligonucleotide to give rise to pMaLUC2, which thereby acquired an extra *Nco*I restriction site overlapping the start codon of *luc*.

The *psa* promoter for the *spa* gene was isolated from plasmid pRIT21 (21) and subjected to site-specific mutagenesis through several rounds of recloning of the *psa* promoter in plasmid pMa58 (19). The resulting plasmid was called pPSA2 and carried one extra *Spe*I and one extra *Afl*III restriction site when compared with pRIT21. The *Spe*I site was introduced with a 38-mer mutator oligonucleotide and was positioned immediately upstream of the presumed SD sequence of *spa* (22). The *Afl*III restriction site was created with a 41-mer oligonucleotide that also introduced an ATG start codon for *spa* by replacing the wild-type TTG. The 5′ end of the *luc* gene was joined to the *psa* promoter by subcloning the 0.5-kb *Eco*RI-*Nco*I fragment isolated from pMaLUC2 in the *Afl*III-*Eco*RI-opened plasmid pPSA2. The resulting plasmid was named pPSA LUC1. The entire *luc* gene was then restored by ligating the 4.8-kb *Afl*III fragment from pPSALUC1 to the 4.9-kb *Afl*III fragment from pSVOAΔL5. The resulting plas-

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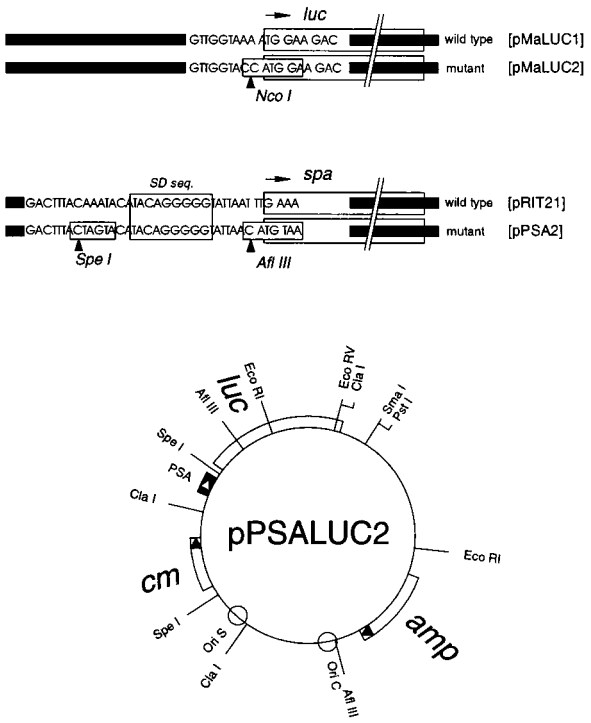

FIG. 1. Diagram of the *P. pyralis luc* expression plasmid pPSALUC2 and schematic representation of mutated sequences introduced by site-directed mutagenesis.

mid pPSALUC2 is suitable for propagation in *S. aureus* as well as in *E. coli* by the presence of appropriate origins for replication, derived from pC194 and pBR322, respectively, and selection markers (*cm* and *amp*) for selection on chloramphenicol- and ampicillin-containing media.

Light measurement. Light was measured in a Labsystems (Helsinki, Finland) Luminoskan at 25°C. The light intensities were registered as relative light units (1 relative light unit = 10^6 photons per s [pps]).

Immobilization of proteins. For the applications reported here, the best binding and blocking results were obtained with black Maxisorp microtiter plates, custom made by Nunc-Inter Med (Roskilde, Denmark), and by incubating protein solutions in phosphate-buffered saline (PBS; 0.01 M P_i–0.15 M NaCl [pH 7.5]) overnight at 4°C. Analyte proteins were incubated in wells containing 50 μl of solution each. Blocking was performed with a 200-μl solution of 1% bovine serum albumin (BSA) in PBS or a 200-μl solution of 0.4% casein in PBS in each well. All washing steps involved three washings with 50 μl of PBS per well. Except for blocking, all further incubations were done in wells containing 50 μl of PBS solutions each. A final washing was performed with PBS or PBS containing 1% Triton X-100.

Production and purification of HBc particles. *E. coli* DH1(pcI857; pPLc3-2/1) (13, 16) was grown in 15 liters of LB medium at 28°C in a Bioflo IV fermentor (New Brunswick, N.J.). Upon induction by a temperature shift to 42°C, the strain produces cytoplasmic hepatitis B core (HBc) particles. The cells were collected with a contifuge (Heraeus), resuspended in 100 ml of 10 mM Tris-HCl (pH 7.5)–10 mM KCl–5% glycerol–0.01% NaN_3, and disrupted with a French press. The crude mixture (10 ml) was centrifuged at a low speed to remove the debris. The supernatant was centrifuged for 16 h in a Beckman ultracentrifuge with a SW41 rotor. The pellet was resuspended in 2 ml of water, loaded on a 20 to 60% sucrose gradient, and centrifuged for 4 h at 100,000 × *g* in a Beckman SW28 rotor. Sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) and Coomassie brilliant blue staining were used to identify those fractions which contained HBc particles of adequate purity.

RESULTS

Stability of pPSALUC2 in *S. aureus*. Plasmid pPSALUC2 contains a fusion between the expression signals for *S. aureus* protein A (*psa*) and the structural gene for *P. pyralis* luciferase (*luc*) engineered in such a way that the relative distances between the *psa* promoter, the *spa* Shine-Dalgarno sequence, and the downstream gene are the same as in the wild-type protein A expression unit. The only alterations in the 5′ untranslated region of *luc* compared with that of the wild-type *spa* are a transition at position −23 and transversions at positions −21 and −1. The Luc polypeptide was synthesized constitutively and was easily detected by Coomassie brilliant blue staining in the soluble protein fraction of *S. aureus* Cowan I(pPSALUC2) (Fig. 2). The constitutive synthesis of the protein did not appreciably affect the growth rate of the recombinant strain. Also, the plasmid was stably inherited, as no differences in the intensities of emitted light signals (see below) could be detected after some 35 generations of growth of the culture under nonselective conditions and all viable cells could be shown to have retained the plasmid (judged by plating on selective agar).

Kinetics of light production by strain Cowan I(pPSALUC2). Saturated LB cultures of the *S. aureus* strains Cowan I(pPSA2) and Cowan I(pPSALUC2) were washed and resuspended in equal volumes of fresh LB medium and buffered with 50 mM sodium citrate at pH 6.9. At zero time, luciferin and Triton X-100 were added to final concentrations of 40 μg ml^{-1} and 1%, respectively. As expected, virtually no emission of light could be detected in Cowan I(pPSA2). Cowan I(pPSALUC2), on the other hand, showed a high initial peak of about 3×10^9 pps. After the addition of substrate, the light level dropped within the first 3 min to a steady intensity of about 10^9 pps, which persisted for at least 30 min (Fig. 2). Analogous results were obtained for *E. coli* MC1061(pPSALUC2), although in general absolute values for light emission were much lower (data not shown). In all subsequent experiments involving the use of Cowan I(pPSALUC2) as a reporter for the presence of IgG molecules, light intensities were measured at the plateau.

Specific immobilization of the engineered bacteria at IgG or antigen-IgG complexes. A fresh colony of *S. aureus* Cowan I(pPSALUC2) was inoculated in 5 ml of selective LB medium and incubated overnight at 37°C with vigorous shaking. The cells were washed and resuspended in 5 ml of PBS. Of this suspension, 50 μl was transferred to microtiter plate wells to which 250 ng of an affinity-purified rabbit IgG fraction was adsorbed and which had been blocked with 1% BSA in PBS.

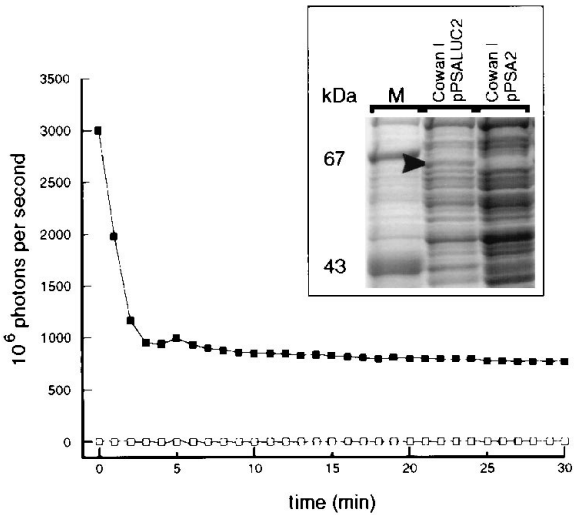

FIG. 2. Light production kinetics. □, Cowan I(pPSA2); ■, Cowan I(pPSA LUC2). The inset shows sections of the protein profiles of the strains Cowan I(pPSA2) and Cowan I(pPSALUC2) revealed by SDS–12.5% PAGE and Coomassie briliant blue staining. The arrowhead indicates the postulated *P. pyralis* Luc polypeptide. Lane M, molecular mass markers.

TABLE 1. Light intensities of *S. aureus* Cowan I(pPSALUC2)[a]

Incubation time (h)	Light intensity of:		Signal/background ratio
	IgG^+ signal (10^6)	IgG^- background (10^6)	
0	39	0.07	558
2	183	0.09	2,033

[a] *S. aureus* Cowan I(pPSALUC2) cells were applied to a substrate to which rabbit IgG molecules had (IgG^+) or had not (IgG^-) been preadsorbed. The signal and background light intensities are expressed as photons per second.

After the cells were washed four times with 0.1% Triton X-100 in PBS and then rinsed two times with PBS, buffered LB medium containing 40 μg of luciferin ml^{-1} and 1% Triton X-100 was added. Wells to which no rabbit IgG had been adsorbed were treated in parallel. A significant level of binding, dependent on the presence of the IgG fraction, could be observed (Table 1). It is of some importance to note that for the applications reported here, the best binding and blocking results (up to 50 times higher than those obtained with microtiter plates from other manufacturers) were obtained with black Maxisorp microtiter plates (Nunc-Inter Med).

In order to investigate whether *S. aureus* Cowan I(pPSA LUC2) could serve as a reporter for more complicated types of immunodetection, a prototype assay for the detection of anti-HBc antibodies in crude human serum was developed. Recombinant HBc particles were prepared from *E. coli* DH1(pcI857; pPLc3-2/1) as described in Materials and Methods. The purified core particles were adsorbed to a microtiter plate in the amount of 75 ng per well. After wells were blocked with 0.4% casein in PBS, a dilution series of crude serum of a patient who had recently recovered from hepatitis B was applied. Although the antibodies could be detected in a single-step reaction (indicating direct contact with the reporter bacteria; data not shown), better results were obtained when secondary antibodies, a cocktail of rabbit IgG antibodies directed against human IgA, IgG, and IgM molecules, were added at a concentration of 2 μg ml^{-1} prior to administration of Cowan I(pPSALUC2) cells grown and pretreated as described above. A clear signal could be detected only when all components of the assay were present. Negative controls included omitting different steps in the buildup and showed, for instance, that a slight cross-reaction occurs between the fraction containing rabbit IgG antibodies directed against human IgA, -G, and -M and the preparation of HBc particles used. This does not, however, impair the validity of the reporter system described here. The results of this experiment are shown in Fig. 3A. Figure 3B shows a reference experiment in which comparable stacks of antigens and antisera were detected with alkaline phosphatase-conjugated goat IgG raised against rabbit IgG (Dako, Glostrup, Denmark) at 1 μg/ml in combination with 4-methoxy-4-(3-phosphatephenyl)spiro-(1,2-dioxetane-3,2′-adamatane (Lumigen PPD; Boehringer). Reactions were performed as directed by the manufacturer. Clearly the detection system using live *S. aureus* Cowan I(pPSALUC2) displays a sensitivity comparable to those of conventional enzymatic methods.

Biological amplification of the light signal. Subsequent to the five final washing steps, LB medium was added to *S. aureus* Cowan I(pPSALUC2) cells which had been immobilized on a microtiter plate coated with rabbit IgG molecules, as described above. The plate was incubated at 37°C for 2 h, and luciferin, buffer, and Triton X-100 were added to the final concentrations described above. We could clearly demonstrate a specific gain in the signal. The gain was reflected not only in absolute numbers but also in an improved ratio between the IgG-specific signal and background. Longer incubations resulted in greater light intensities but did not improve the signal/background ratio (Table 1).

DISCUSSION

Our results indicate that live bacteria, expressing the *P. pyralis luc* gene, can be used as sensitive detection tools in immunodetection. In our study, an engineered strain of *S. aureus* Cowan I was used.

The kinetics of light production displayed by strain *S. aureus* Cowan I(pPSALUC2) typically show a high peak value at the beginning of the curve which decreases within 3 min to a plateau. The height of the plateau was regarded as a standard for the efficiency of the tested system, this being a sum of the luciferin uptake level, the O_2 concentration, the ATP content of the cell, the level of quenching of the emitted light, and the overall growth conditions. Under the conditions used, the

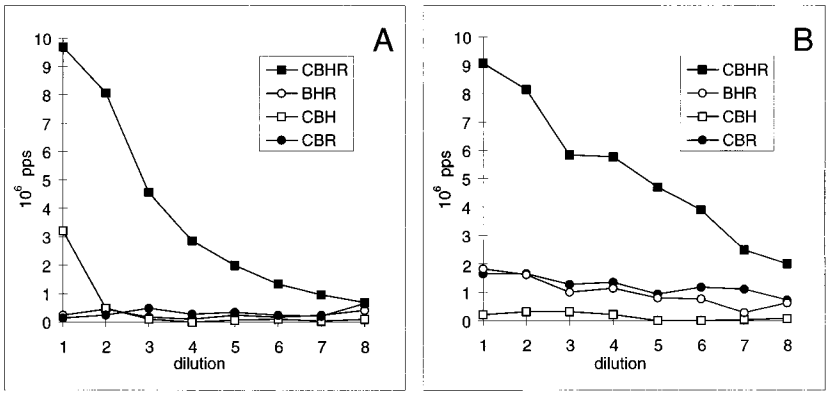

FIG. 3. (A) *S. aureus* Cowan I(pPSALUC2)-based detection of anti-HBc activities in crude human sera. (B) Detection, based on alkaline phosphatase-conjugated goat IgG raised against rabbit IgG and Lumigen PPD, of anti-HBc particle activities in crude human sera. Consecutive layers used in the test were as follows: C, purified HBc particles (75 ng per well); B, blocking (0.4% casein in PBS); H, human serum with anti-HBc particle activity (dilution 1 = 1:50 dilution of the crude material in PBS; further dilutions increase by 1:2); R, rabbit IgG antibodies directed against human IgA, IgG, and IgM (100 ng per well). These results were recorded 5 min after the addition of the substrates luciferin (A) and Lumigen PPD (B).

height of the plateau was 10^9 pps for *S. aureus* Cowan I(pPSALUC2).

In recent years multiple techniques based on the detection of emitted light have been developed for immunodetection. These often proved to be very sensitive when compared with other existing methods. In previous work (20) we observed that *S. aureus* Cowan I cells could be immobilized specifically on polystyrene surfaces on which IgG molecules had been adsorbed. By propagating in this strain the *psa-luc* expression cassette described above, we were able to specifically bind these light-producing cells to antigen-antibody complexes and thus perform immunodetection with a live reporter tool. One microtiter well filled with 50 μl of solution can bind approximately 10^8 spheres with a radius of 1 μm when packing is optimal. A saturated *S. aureus* culture contains about 2×10^9 cells and produces 10^9 pps. Theoretically the maximum light production observed after perfect binding should therefore be about 5×10^7 pps, which fits well with the observed 3.9×10^7 pps. This indicates that the observed signal can be interpreted quantitatively.

The signal which was obtained from this assay could further be amplified fourfold by in situ incubation of the bound cells in growth medium. A prerequisite for the use of this amplification step, however, is the achievement of a very low background level.

We further demonstrated the applicability of this reporter system in more complicated setups. For this purpose we performed an assay in which we attempted to detect anti-HBc antibodies in crude human serum. The cross-reactions among some components of the detection stack limited the sensitivity to some extent. This does not, however, impair the utility of *S. aureus* Cowan I(pPSALUC2) as a reporter for secondary or tertiary antibodies. Moreover, we could demonstrate that the detection system described here displays a sensitivity comparable to that of detection based on Lumigen PPD used in combination with an appropriate alkaline phosphatase-conjugated antibody.

In summary, the new reporter system based on live bacteria allows one to indirectly bind large quantities of luciferase, together with an ATP-generating system, to immobilized IgG molecules. Because of the high level of stability of light emission (of the glowing type) over time, the method is well suited for quantitative measurements of analytes in a large number of samples and should be amenable to automation. In this respect it compares favorably with enzymatic detection methods which are of the flash-emitting type. Since the expression unit neither kills nor imposes severe growth restrictions on the cells in which it is brought to function, cells which are bound at a surface go on multiplying, thus providing an elegant method for biological amplification of a primary, weak signal. This finding is new and is an exclusive property of immobilized living bacteria. In this way the presented material opens broad perspectives for an alternative, up-to-now-unexplored type of signal enhancement.

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