SHORT COMMUNICATIONS

A Turbidimetric Method for the Assay of Pyocin Activity

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Bacteriocins have been the subject of recent reviews (Reeves, 1965*a*; Nomura, 1967; Bradley, 1967); the last author suggested that bacteriocins might be classified according to whether they are of low or high molecular weight. The most studied pyocins are the high molecular weight type which have been shown to closely resemble T-even coliphage tails in size and morphology and consist of a contractile sheath and inner core (Ishii, Nishi & Egami, 1965; Higerd, Baechler & Berk, 1967, 1969; Bradley, 1967). The bacterial strain described in this communication produces a pyocin which resembles this type.

No rapid and quantitative method has been described for assaying pyocin activity. The pyocin activity in a broth culture may be determined semi-quantitatively by lawn-dilution methods (Paterson, 1965; Higerd *et al.* 1969). Drops of such a broth culture, appropriately diluted, are spotted on nutrient agar plates the surface of which has been seeded with an organism sensitive to the particular pyocin under assay. After incubation the highest dilution of the pyocin preparation capable of preventing growth of the sensitive strain is termed the pyocin titre and is often expressed in arbitrary units (a.u.): an a.u. being the reciprocal of the pyocin titre. Unlike the lawn-dilution assay for phages where one phage particle can give rise to a plaque, one bacteriocin particle does not give rise to an area of inhibition since bacteriocins kill sensitive cells without being reproduced within them. Consequently at high dilutions the number of pyocin particles per drop is insufficient to produce a discernible area of inhibition on the seeded plate. The graduation from confluent growth to complete inhibition with increasing concentrations of pyocin and the need to use many pyocin dilutions make it both difficult and laborious to determine pyocin titres accurately by this method.

A turbidimetric method for assaying a small, low molecular weight colicin was described by Reeves (1965*b*). This communication shows that a turbidimetric assay method which is both simple and accurate is suitable for the phage tail-like bacteriocin pyocin.

METHODS

Organisms. *Pseudomonas aeruginosa* strain R2I, a non-lysogenic type I pyocin producer (Gillies & Govan, 1966), and indicator strain I, sensitive to type I pyocin, were obtained from Dr J. R. W. Govan, Bacteriology Department, Edinburgh University.

Media. (a) MSYE: (% w/v) glucose, 0.2; K₂HPO₄, 0.2; NH₄H₂PO₄, 0.3; yeast extract (Oxoid), 0.05, pH 6.8. MgSO₄·7H₂O, 0.1 (added after separate sterilization). (b) Assay broth: nutrient broth no. 2 (Oxoid), 7 vol.; 0.3 M-glucose in distilled water, 4 vol.; 0.25 M-tris/HCl buffer pH 7.6, 6 vol. These three solutions were used unsterilized within a day or two of preparation.

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Preparation of indicator bacteria. Indicator strain 1 was grown in 100 ml. MSYE medium in a 350 ml. Erlenmeyer flask for 16 h. at 30° in an orbital incubator (Gallenkamp) operated at 150 rev./min. The cells were harvested by centrifugation and suspended in 0.9 % (w/v) KCl to give an extinction of 60 units at 450 nm. in an EEL Spectra (Evans Electro-selenium Ltd). Such a suspension was found to contain approximately 15×10^8 viable organisms/ml. and to have a dry weight of 0.4 mg./ml.

Preparation of type I pyocin. Strain R21 was grown in 100 ml. MSYE under the same conditions used for the indicator cells. The cells from 50 ml. of culture were harvested by centrifugation and resuspended in 100 ml. fresh MSYE medium. Mitomycin C (Sigma) was added to a final concentration of 1.5 µg./ml. and the culture incubated with vigorous shaking for 6 h. at 37° in a reciprocating water bath. The cells were then removed by centrifugation and the opalescent supernatant containing pyocin was sterilized with chloroform.

Composition of assay system. Assay broth, 1.7 ml.; pyocin preparation (appropriately diluted in assay broth) or assay broth, 0.3 ml.; indicator cell suspension (0.4 mg. dry wt of organisms/ml.), 1 ml.

Assay method. The assay broth was dispensed into 18 test tubes (6 in. $\times$ $\frac{5}{8}$ in.). To duplicate tubes, 0.3 ml. of each pyocin dilution was added: the four remaining tubes received 0.3 ml. of assay broth. The tubes were incubated for 15 min. at 37° in a water bath and at 1.5 min. intervals 1 ml. of indicator cell suspension (similarly incubated) was added to each tube. Two of the tubes containing no pyocin were removed immediately following the addition of cells and the extinction values of the contents measured at 650 nm. against a distilled water blank using the EEL Spectra. The remaining tubes were incubated at 37° in a reciprocating water bath operating at 170 strokes/min. (1.5 in. stroke length). The tubes were removed in sequence after incubation for 60 min. and the extinction values of their contents measured at 650 nm. without delay. In order to maintain a constant shaking speed during incubation, the tubes were removed without stopping the apparatus and replacement test tubes containing 3 ml. of water were substituted for each assay tube as it was removed.

Viable counts. The Miles & Misra (1938) technique was used; counts on nutrient agar were made after incubation for 16 h. at 37°.

RESULTS

The results for a typical pyocin assay are shown in Table 1.

A curve similar to a normal sigmoid curve is obtained when the $\log_{10}$ of the pyocin dilution is plotted on the abscissa and the extinction on the ordinate. Such a curve can be converted to a straight line by probit transformation (Finney, 1952). Probit 5 represents the mean of the normal distribution from which the straight line was obtained and is therefore an accurate measure of the 50 % response.

From the data given in Table 1 it can be graphically shown that the $\log_{10}$ pyocin dilution corresponding to probit 5 is -2.7 , which represents a pyocin dilution of $\frac{1}{500}$: the original pyocin preparation can therefore be said to have a potency of 500 probit units/ml. (p.u./ml.).

Viable counts made on the contents of the pyocin assay tubes after the 60 min. incubation period invariably showed that the percentage survival of the initial inoculum subjected to probit analysis provided a straight line plot and in this particular example that probit 5 again corresponded to a $\log_{10}$ pyocin dilution of -2.7 . Therefore under the conditions of the turbidimetric assay, the measurement of extinction (650 nm.) can be used as a rapid and accurate measure of the 50 % lethal dose for the indicator cell inoculum.

Table 1. *Probit analysis of turbidimetric pyocin assay data*

Percentages were converted to probits by means of a conversion table (Finney, 1952).

Log ₁₀ pyocin dilution	E _{650nm} (1 h.)	Increase in E from the zero time value (14)	% of control increase	Probit
-2.3	15	1	8	3.59
-2.6	19.25	5.25	42	4.8
-2.9	23.75	9.75	78	5.77
-3.0	24.25	10.25	82	5.92
-3.3	26	12.0	96	6.75
-3.6	27.5	13.5	108	—
-3.9	26.5	12.5	100	—
Control (no pyocin)	26.5	12.5	100	—

DISCUSSION

The results show that a turbidimetric method can be used to assay the large molecular weight bacteriocin pyocin. This method is simpler to perform and is both more rapid and more accurate than the lawn-dilution method. By subjecting the results to probit transformation a standard unit of pyocin activity is obtained: this, the probit unit (p.u.), can be compared directly to the number of cells killed by that amount of pyocin. In contrast the results of the lawn-dilution method expressed either as pyocin titre or arbitrary units of pyocin cannot be related to the number of cells killed. In the turbidimetric method the effect of the pyocin is measured objectively and is not dependent on personal judgement. Indicator cell suspensions and incubation conditions are easily standardized and this should allow comparison of results between laboratories. Results can be obtained within 2 h. thus providing a check on the activity of fractions in a purification scheme before proceeding to the next step.

Reeves (1965*b*), using a similar assay system, assumed that the effect of colicin was immediate, killing a certain percentage of the cells, and that the rise in extinction during incubation was proportional to the numbers of bacteria which survived. This assumption appears to be valid for the pyocin assay method.

Vibriocin (the bacteriocin produced by *Vibrio cholerae*) appears from its description by Jayawardene & Farkas-Himsley (1969) to be similar to pyocin in both size and morphology. These authors determined vibriocin activity by incubating a saline suspension of logarithmic phase indicator cells with a sample of vibriocin for 30 min. at 37° and determining the proportion of survivors by means of viable counts. A similar method was used by Seaman, Tarmy & Marmur (1964) to assay the mitomycin C inducible defective phages of *Bacillus subtilis* (termed PBSX particles) which, like bacteriocins, do not reproduce in sensitive cells. The turbidimetric method described for pyocin assay might also be applicable to these two systems.

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