Increased Numbers of Heat-resistant Spores Produced by Two Strains of *Clostridium perfringens* Bearing Temperate Phage s9

By ADELLE W. STEWART* AND MICHAEL G. JOHNSON

Departments of Food Science and Microbiology, Clemson University,
Clemson, South Carolina 29631, U.S.A.

(Received 23 June 1977)

Sporulation kinetics and spore heat resistance data were compared for a lysogenic strain of *Clostridium perfringens*, s9, before and after curing with ultraviolet irradiation. The cured strain showed the same growth rate in broth media as the lysogenic strain but took 6 h longer to form refractile spores. For lysogenized and cured strains the percentages of refractile spores produced that were heat-resistant (80 °C for 15 min) were 50 and 0.2, respectively. When reinfected with the temperate phage, the cured strain produced spores in 2 to 3 h, like the original lysogenic culture, and 10 % of the spores produced were heat-resistant.

INTRODUCTION

Lysogeny in *Clostridium perfringens* was first demonstrated by Smith (1959) who characterized several temperate phages. Mahony & Kalz (1968) isolated and characterized a temperate phage from cells of a lysogenic strain of *C. perfringens*, s9, which they induced with ultraviolet light. This phage showed incomplete lysis of broth cultures with latent and release periods of 45 min each in indicator strain s13. Maximum titres of this phage were 1×10^4 to 1×10^5 plaque-forming units (p.f.u.) ml⁻¹. A second isolated phage (Mahony & Easterbrook, 1970) caused complete lysis of broth cultures of host cells and produced somewhat higher phage titres of about 1×10^6 p.f.u. ml⁻¹.

No studies of effects of bacteriophages on spore production by *C. perfringens* have, to our knowledge, been reported. Duncan, Strong & Sebald (1972) reported that the temperate phages they tested apparently did not affect enterotoxin production. Any such information would be very useful because these workers have presented convincing evidence that *C. perfringens* produces a protein enterotoxin during sporulation. Certain strains during sporulation apparently overproduce a spore coat protein that is antigenically like the enterotoxin. Sporulation-negative mutants produced little or no enterotoxin (Duncan, 1973; Duncan, King & Friebe, 1973). Their evidence therefore suggests that any treatment which reduces sporulation of *C. perfringens* will also reduce enterotoxin production and hence decrease the organism's ability to cause foodborne illness.

In vitro studies with strains of *C. perfringens* generally show relatively low spore to bacterium ratios of 0.01 to 0.10. If the model of Duncan *et al.* (1972) is correct, however, this organism must sporulate well *in vivo* since it is one of three major causes of bacterial foodborne gastroenteritis in the United States.

With this apparent paradox in mind, we examined the sporulation characteristics of two *C. perfringens* strains lysogenized or cured of bacteriophage. The hypothesis tested

* Present address: Department of Natural Sciences, South Carolina State College, Orangeburg, South Carolina 29115, U.S.A.

was whether the presence or absence of phage in host cells exerted any effects on sporulation. A preliminary report of this work was presented earlier (Stewart & Johnson, 1975).

METHODS

Bacterial and bacteriophage strains. The strains of *C. perfringens* used were s9, a lysogenic strain, and s13, an indicator strain, both obtained from Dr D. E. Mahony, Dalhousie University, Halifax, Nova Scotia, Canada. Strain s9 when cured of its prophage, s9c, with ultraviolet (u.v.) light (Mahony & Kalz, 1968) was designated as s9c. Phage s9 produced a lytic effect on s9c. When surviving cells of s9c in or near plaques were transferred to tryptone/yeast extract/glucose broth (TYG broth; Bioquest, BBL) and incubated for 5 days at 30 °C, phages were detectable by u.v. induction. One such strain after isolation and purification was designated s9CR and used in further studies. Strain s13 when lysogenized with prophage s9 was designated s13i.

Media. All strains of *C. perfringens* were maintained in Brewer's cooked meat medium (Difco). Thioglycollate broth (Bioquest, BBL) was used for vegetative growth, and the medium of Duncan & Strong (1968) (DS medium) was used for spore production. Host strains for phage production were grown in Brain Heart Infusion broth (Difco) containing 0.1 % (w/v) sodium thioglycollate (BHI broth).

Growth conditions. All cultures, unless stated otherwise, were grown at 37 °C. Oxygen was exhausted from liquid media by heating culture tubes in a boiling water bath for 5 min and then cooling them before inoculation. Bacteria and spores were counted on solid media by the Gas Pak method (Bioquest, BBL) or the pouch method of Bladel & Greenberg (1965). Pouches were made by sealing together two layers of plastic laminate film (R460, W. R. Grace Co., Cryovac Division, Duncan, South Carolina, U.S.A.) having low oxygen permeability. Total bacteria and refractile spore counts were determined microscopically with a Petroff-Hauser counting chamber and a phase contrast microscope (Zeiss Standard Model RA) by the method of Meynell & Meynell (1970). Growth of strains of *C. perfringens* was measured using matched 13 × 100 mm screw-capped test tubes, containing 6 ml thioglycollate medium inoculated with 0.1 ml of a culture grown in this medium at 37 °C for 4 h. These cultures were incubated at 37 °C and growth was monitored hourly, for 12 h, by following changes in absorbance with a Turner Model 330 spectrophotometer at 600 nm.

Induction of prophage s9. *Clostridium perfringens* s9 grown in BHI broth for 6 h was centrifuged at 22 °C for 10 min in an I.E.C. HN-S centrifuge at 4500 rev. min⁻¹. The resulting pellet was washed twice by centrifugation and resuspended in 0.85 % (w/v) NaCl. Washed suspension (5 ml) was placed in an open, sterile Petri dish and exposed to two 15 W General Electric (G15 T8) germicidal lamps at a distance of 38 cm for 100 s. After irradiation, 1 ml samples were placed in 9 ml BHI broth and incubated for 150 min, then centrifuged to pellet the organisms. Supernatant fluid was assayed for p.f.u. on lawns of indicator strain s13 by the double layer method of Adams (1959). The base layer used was the medium of Mahony & Kalz (1968) and the overlay was 0.6 % (w/v) Noble agar (Difco).

Curing of lysogenic strain s9. *Clostridium perfringens* strain s9 (0.1 ml, containing 10⁵ exponential-phase organisms) was spread on the surface of a plate of the agar medium of Mahony & Kalz (1968). The inoculated plate was irradiated with the two u.v. lamps described above for 60 s at a distance of 20 cm, and then incubated in a Gas Pak jar for 24 h. Survivors formed two types of colonies; they either had a turbid clearing zone in the centre or they did not have such a zone. Colonies of the former type were transferred to BHI broth and incubated at 37 °C. When cultures reached their exponential phase of growth they were again irradiated with u.v. light to induce any prophage still remaining. Induced organisms showing no detectable phages when spotted on lawns of s13 were considered to be cured of the prophage s9 and designated s9c. This was confirmed by the method of Siddiqui *et al.* (1974), using mitomycin C (1 µg ml⁻¹).

Lysogenization of the cured strain s9c and indicator strain s13. Strains s9c and s13 were each plated with phage s9. The plated cultures were incubated in a Gas Pak jar for 24 h and observed for plaques. Cells near the edges of and within plaques were picked with a platinum inoculating needle, transferred to TYG broth and incubated at 30 °C for 5 days. Cultures which developed from such plaque areas were shown to harbour the prophage s9 using the u.v. induction procedure described above and lawns of the indicator strain s13. The relysogenized strain of s9c was designated as s9CR, and the lysogenized strain of s13 as s13i.

Sporulation. A vegetative culture was obtained by inoculating 0.1 ml of a culture from cooked meat medium into 10 ml thioglycollate medium and incubating at 37 °C for 16 h. Two subsequent 1 ml serial transfers were made into thioglycollate medium (10 ml) and each was incubated for 4 h. The third culture was then centrifuged at 22 °C in an I.E.C. HN-S centrifuge at 4500 rev. min⁻¹ for 10 min. The cell pellet was suspended in DS medium (10 ml) which had previously been heated in a boiling water bath for 5 min and cooled to 37 °C. Cultures were incubated at 37 °C for 24 h. To determine the number of heat-resistant

Table 1. *Comparison of numbers of refractile and heat-resistant spores produced by lysogenic and cured strains of C. perfringens in DS medium*

Strain	Max. no. of refractile spores (ml ⁻¹)	Time for max. no. of refractile spores (h)	Percentage of refractile spores that were heat-resistant	Time for max. no. of heat-resistant spores (h)
s9	1×10^7	3	50.0	3
s9C	1×10^6	9	0.2	9-10
s9CR	2×10^7	4	10.0	4
s13	ND	—	—*	8
s13I	1×10^7	3	2.0	5-6

ND, Not detectable.

* Not determinable; 5×10^5 heat-resistant spores ml⁻¹ were present by 8 h.

spores, suspensions in tubes were heated in an oil bath at 80 °C for 15 min, diluted with 0.1 % (w/v) peptone, plated on Brewer's anaerobic agar (Difco) and incubated in Gas Pak jars for 48 h at 37 °C.

After sporulation, cultures were harvested as above. Their dipicolinic acid (DPA) content was determined by the method of Janssen, Lund & Anderson (1958) using pellets from non-sporulated cultures as controls. DPA used to prepare standard curves was purchased from Sigma. Pellet dry weights were determined after drying samples of cultures in aluminium dishes to constant weight at 100 °C.

Data presented are the average values of duplicate experiments.

RESULTS

Strain and phage characteristics

The growth rate constants (k , expressed as the number of doublings per hour) for the lysogenic strain s9, the cured strain s9C and the relysogenized strain s9CR in thioglycollate broth were similar, being 1.2 to 1.3 h⁻¹. The values of k for the indicator strain s13 and the lysogenized strain s13I were 1.0 to 1.1 h⁻¹. Strains s9CR and s13I, unlike their respective parent strains, had lag periods of 2 to 3 h before exponential growth started. All the s9 strains had similar mucoid colonies.

On lawns of s13, phage s9 produced turbid plaques that had irregular edges and an average diameter of 1 mm. These small plaques were difficult to see unless viewed against a dark background with oblique lighting.

Complete lysis of *C. perfringens* s13 by s9 was never observed in liquid media although there was always some increase in phage titre. Serial transfers of phage lysate to fresh cultures of s13 increased the phage concentration to no more than 1×10^5 p.f.u. ml⁻¹. Apparently, the bacteria remaining in the culture either became resistant to phage adsorption and infection or became lysogenized.

Spore production

The total microscopically-refractile and heat-resistant spore counts produced by the lysogenized strain s9, the cured strain s9C and the reinfected strain s9CR in DS medium were compared. Strain s9 produced maximum numbers of refractile spores (1×10^7 ml⁻¹) by about 3 h compared with 9 h for s9C and 4 h for s9CR (Table 1). While strains s9, s9C and s9CR required corresponding periods (3, 9 to 10, and 4 h) to form maximum numbers of heat-resistant spores, the percentages of refractile spores that were heat-resistant differed, being 50, 0.2 and 10, respectively.

Cultures of the indicator strain s13 initiated sporulation in 3 to 4 h and produced maximum numbers of heat-resistant spores (about 5×10^5 ml⁻¹) by 8 h (Table 1). Since refractile spores were apparently produced in numbers too low to be enumerated microscopically, even after 12 h, the percentage of spores produced that were heat-resistant could not be calculated.

Table 2. *Dipicolinic acid content of sporulated suspensions produced by lysogenic and cured strains of C. perfringens*

Strain	DPA content [$\mu\text{mol (g dry wt bacteria)}^{-1}$]
S9	2.7
S9C	1.2
S9CR	2.2
S13	4.7
S13I	2.6

Strain S13I, i.e. strain S13 lysogenized with phage s9, initiated sporulation in 1 to 2 h but required 5 to 6 h to produce maximum numbers of heat-resistant spores ($5 \times 10^5 \text{ ml}^{-1}$) (Table 1). Maximum numbers of refractile spores ($1 \times 10^7 \text{ ml}^{-1}$) were observed microscopically after 3 h and 2 % of these were heat-resistant.

Dipicolinic acid content of spores

Expressed as $\mu\text{moles per g dry weight bacteria}$, the amounts of DPA in the endospores produced by the five strains varied little, ranging from 1.2 for strain S9C to 4.7 for strain S13 (Table 2).

DISCUSSION

Clostridium perfringens, unlike many other clostridia and most bacilli, sporulates relatively poorly in laboratory media. We were interested to see whether the presence of prophages in cells affected sporulation in this organism.

Strain S9CR, the cured strain into which the prophage s9 was reintroduced, produced maximum numbers of spores in about 5 to 6 h less time than did S9C (Table 1) and the percentage of heat-resistant spores was increased from 0.2 to 10 %. Similarly, strain S13I, the indicator strain into which the prophage s9 was introduced, produced maximum levels of heat-resistant spores in 2 to 3 h less time than did S13. These data argue that when temperate phage s9 was present in host strains of *C. perfringens*, it could somehow alter the way the genetic information for sporulation was read. One possibility is that this phage genome itself contained some genetic information that affected host cell sporulation, a mechanism known as 'phage conversion' (Hayes, 1968). A classic example of this mechanism is the toxigenicity which lysogenization with phages of the β group confers on *Corynebacterium diphtheriae* (Freeman, 1951; Freeman & Morse, 1952). A second possibility is that phage s9, when induced out of host cells, transferred host sporulation-specific genes to recipient cells, a mechanism known as phage transduction (Zinder & Lederberg, 1952). Before one can unequivocally judge which of these mechanisms is operating in this prophage-host system, genetic markers in *C. perfringens* coding for non-sporulation specific functions but close to those for sporulation will have to be mapped. In the interim, the high frequency with which sporulation was altered as the prophage was removed from or reinstated in cells argues in favour of the phage conversion hypothesis. This hypothesis is strengthened by the data of Grant & Riemann (1976) who found that three temperate phages isolated from lysogenic strains of *C. perfringens* Type C were apparently unable to transduce erythromycin resistance.

Until recently, dipicolinic acid was thought necessary for heat resistance in spores of clostridia and bacilli (Murrell, 1969). However, data challenging this concept have been reported. Spores of two mutant strains of *Bacillus cereus* and *Bacillus subtilis* which lacked any detectable DPA were nevertheless found to be quite heat-resistant (Hanson *et al.*, 1972; Zytkevich & Halvorson, 1972). Additional work disputing the role of DPA in spore heat resistance was reported recently by Imae & Strominger (1976*a, b*). They found that the development of spore heat resistance in mutant strains of *Bacillus sphaericus* and

B. subtilis was more closely associated with the accumulation in the cortex region of 'muramic lactam', not DPA. We were unable to detect differences in the DPA content of sporulated suspensions of *C. perfringens* which could explain the differences in their heat resistance.

Unlike the positive effect exerted on sporulation by phage s9 which we report here, several recent studies indicate that prophages can affect sporulation negatively. Hendry, Gillespie & Fitz-James (1976) found that sporulation proceeded faster in mutants of *Bacillus medusa* from which the phage ϕ med-2 was removed by u.v. light or mitomycin C treatment. Similar phage or plasmid-associated repression of sporulation in *Bacillus pumilus* has been reported (Lovett, 1973; Bramucci & Lovett, 1974; Lovett & Bramucci, 1974).

This report is, to our knowledge, the first showing that sporulation and spore heat resistance in *C. perfringens* can be affected by phage. It seems obvious that caution should be exercised in attempting to interpret the results of sporulation experiments with *C. perfringens* Type A strains in which the possibility of lysogeny has not first been checked. While Sebald & Costilow (1975) were unable to detect any transduction, transformation or conjugation mechanisms in several strains of this organism, the minimal medium they developed to detect auxotrophic mutants along with the phage system we report here should prove useful in deciphering the genetic mechanisms controlling sporulation in this organism.

Technical Contribution No. 1391 of the South Carolina Agricultural Experiment Station, Clemson, South Carolina 29631, U.S.A.

This research was supported in part by a fellowship awarded to A.W.S. from the Ford Foundation and the National Fellowships Fund and in part by funds provided to the South Carolina Agricultural Experiment Station for Hatch Project SC000006 from the United States Department of Agriculture. The authors thank Ann W. Baxter, M. J. B. Paynter and S. S. Hayasaka for helpful discussions. This paper was taken in part from a thesis submitted by A.W.S. to Clemson University in partial fulfilment of the requirements of the Ph.D. degree.

REFERENCES

- ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience.
- BLADEL, B. O. & GREENBERG, R. A. (1965). Pouch method for the isolation and enumeration of clostridia. *Applied Microbiology* **13**, 281-285.
- BRAMUCCI, M. G. & LOVETT, P. S. (1974). Temperate bacteriophage infectious for asporogenic variants of *Bacillus pumilus*. *Journal of Virology* **14**, 1281-1287.
- DUNCAN, C. L. (1973). Time of enterotoxin formation and release during sporulation of *Clostridium perfringens* Type A. *Journal of Bacteriology* **113**, 932-936.
- DUNCAN, C. L. & STRONG, D. H. (1968). Improved medium for sporulation of *Clostridium perfringens*. *Applied Microbiology* **16**, 82-89.
- DUNCAN, C. L., STRONG, D. H. & SEBALD, M. (1972). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *Journal of Bacteriology* **110**, 378-391.
- DUNCAN, C. L., KING, G. J. & FRIEBEN, W. R. (1973). A paracrystalline inclusion formed during sporulation. *Journal of Bacteriology* **114**, 845-859.
- FREEMAN, V. J. (1951). Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae*. *Journal of Bacteriology* **61**, 675-688.
- FREEMAN, V. J. & MORSE, I. U. (1952). Further observations on the change to virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae*. *Journal of Bacteriology* **63**, 407-414.
- GRANT, R. B. & RIEMANN, H. P. (1976). Temperate phages of *Clostridium perfringens* Type C. *Canadian Journal of Microbiology* **22**, 603-610.
- HANSON, R. S., CURRY, M. V., GARNER, J. V. & HALVORSON, H. O. (1972). Mutants of *Bacillus cereus* strain T that produce thermoresistant spores lacking dipicolinate and have low levels of calcium. *Canadian Journal of Microbiology* **18**, 1139-1143.
- HAYES, W. (1968). *The Genetics of Bacteria and Their Viruses - Studies in Basic Genetics and Molecular Biology*, 2nd edn. New York: John Wiley.
- HENDRY, G. S., GILLESPIE, J. B. & FITZ-JAMES, P. C. (1976). Bacteriophage and bacteriophage-like structures carried by *Bacillus medusa* and their effect on sporulation. *Journal of Virology* **18**, 1051-1062.
- IMAE, Y. & STROMINGER, J. L. (1976a). Relationship between cortex content and properties of *Bacillus sphaericus* spores. *Journal of Bacteriology* **126**, 907-913.

- IMAE, Y. & STROMINGER, J. L. (1976*b*). Cortex content of asporogenous mutants of *Bacillus subtilis*. *Journal of Bacteriology* **126**, 914-918.
- JANSSEN, F. W., LUND, A. J. & ANDERSON, L. E. (1958). Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127**, 26-27.
- LOVETT, P. S. (1973). Plasmid in *Bacillus pumilus* and the enhanced sporulation of plasmid-negative variants. *Journal of Bacteriology* **115**, 291-298.
- LOVETT, P. S. & BRAMUCCI, M. G. (1974). Biochemical studies of two *Bacillus pumilus* plasmids. *Journal of Bacteriology* **120**, 488-494.
- MAHONY, D. E. & EASTERBROOK, K. B. (1970). Intracellular development of a bacteriophage of *Clostridium perfringens*. *Canadian Journal of Microbiology* **16**, 983-988.
- MAHONY, D. E. & KALZ, G. C. (1968). A temperate bacteriophage of *Clostridium perfringens*. *Canadian Journal of Microbiology* **14**, 1085-1093.
- MEYNELL, G. C. & MEYNELL, E. (1970). *Theory and Practice in Experimental Bacteriology*, 2nd edn. Cambridge: Cambridge University Press.
- MURRELL, W. G. (1969). Chemical composition of spores and spore structures. In *The Bacterial Spore*, pp. 215-274. Edited by G. W. Gould and A. Hurst. New York: Academic Press.
- SEBALD, M. & COSTILOW, R. N. (1975). Minimal growth requirements for *Clostridium perfringens* and isolation of auxotrophic mutants. *Applied Microbiology* **29**, 1-16.
- SIDDIQUI, A., HURT, E., MANDAGERE, U. & GOLDBERG, I. E. (1974). Rapid plate method for the isolation of lysogenic bacteriophages. *Applied Microbiology* **27**, 278-280.
- SMITH, H. W. (1959). The bacteriophages of *Clostridium perfringens*. *Journal of General Microbiology* **21**, 622-630.
- STEWART, A. W. & JOHNSON, M. G. (1975). Effect of a temperate bacteriophage of *Clostridium perfringens* s9 on sporulation and spore heat resistance. *Abstracts of the Annual Meeting of the American Society for Microbiology*, P8, 201. Washington: American Society for Microbiology.
- ZINDER, N. D. & LEDERBERG, J. (1952). Genetic exchange in *Salmonella*. *Journal of Bacteriology* **64**, 679-699.
- ZYTKOVICZ, T. H. & HALVORSON, H. O. (1972). Some characteristics of dipicolinic acid-less mutant spores of *Bacillus cereus*, *Bacillus megaterium* and *Bacillus subtilis*. In *Spores V*, pp. 49-52. Edited by H. H. Halvorson, R. Hanson and L. L. Campbell. Washington: American Society for Microbiology.