THE ERWINIA-COLIFORM RELATIONSHIP

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Erwinia, a genus of bacterial plant pathogens, has long been recognized as closely related to the *Escherichia-Aerobacter* group. This view, although readily accepted, has been the object of little comparative study; the relationship is usually taken for granted, the result being a relatively poor understanding of the similarities of the two groups. On the basis of its disease-producing ability in plants, Bergey *et al.* (1939) gives the group tribal ranking, Erwineae, in the family Enterobacteriaceae. Other than the obvious facts that the species of *Erwinia* are peritrichously flagellated, gram negative, do not form spores and ferment many carbohydrates, including lactose, our knowledge of the relationship is meager. The possibility that the *Erwinia*[1] might be coliform organisms has been given comparatively little thought.

Stuart *et al.* (1938, 1940) recognized the possibility of *Erwinia* cultures being confused with coliform organisms, or vice versa, and included some plant cultures in their studies. Stanley (1939) likewise declared the soft-rot group to belong to the colon-typhoid-dysentery group, and suggested that organisms identified as *Erwinia* might be *Escherichia coli*, the latter interpreted in a broad sense. According to Parr (1939), F. D. Chester stated that the genus *Erwinia* was established on a purely utilitarian basis and had no genetic standing.

Dowson (1939), following the usual British practice, advocated the inclusion of the soft-rot pathogens in the genus *Bacterium* Lehmann and Neumann. By this procedure he recognized the apparent close relationship of the coliform and *Erwinia* groups, as the former are likewise placed in this genus.

Unfortunately, the biochemical characteristics of the soft-rot organisms are as variable as those of coliform isolates (Stanley 1939, Elrod 1941). It has been shown, especially by Stanley, that fermentative shifts are common, tending to throw a culture from one species to another. These changes usually concern a change from an aerogenic to an anaerogenic state, although some variation has been noted in the methyl-red, Voges-Proskauer and citrate tests. Elrod (1941) found that the soft-rot group is as antigenically heterogeneous as the coliform group.

In the light of our abundant knowledge of the coliform group, it was decided first to employ "coliform" methods in an attempt to arrive at a better understanding of the *Erwinia-Escherichia-Aerobacter* relationship. The recent work of Stuart, Mickle and Borman (1940) on aberrant coliforms offers a way of

[1] The term *Erwinia* in this paper is taken to mean the so-called soft-rot group. This group includes: *Erwinia carotovora* (Jones) Holland, *Erwinia aroideae* (Townsend) Holland, *Erwinia solanisapra* (Harrison) Holland and *Erwinia phytophthora* (Appel) Bergey *et al.*

grouping the soft-rot organisms with the Eschericheae. Accordingly, the following tests were performed: 1) lactose fermentation, 2) IMViC (indole, methyl-red, Voges-Proskauer, citrate) plus cellobiose fermentation, and 3) gelatin liquefaction. Our knowledge of the reactions of coliform cultures in these tests was abundant and there was no need to repeat this work.

Moreover, the main criterion of the genus *Erwinia*, i.e., the ability to attack plant tissues, was applied to coliforms. This characteristic is usually associated with the ability of the organisms to breakdown pectin (Chester in Bergey *et al.*, 1939). Why this idea is prevalent is difficult to fathom, unless it arises from a lack of understanding of pectic compounds in the plant. It is known, although not widely recognized, that the enzymes protopectinase (which brings about tissue disintegration) and pectinase (which is associated with the breakdown of pectin or pectic acid) are different entities (Davison and Willaman, 1927). Nevertheless, it was thought advisable to determine whether or not any correlation did exist between the ability to disintegrate vegetable tissue and the fermentation of pectin. If such were the case, we would have a readily accessible method for distinguishing between *Erwinia* and coliform cultures.

Cultures used. The soft-rot isolates used in this study have been tested for rotting ability many times and until January, 1941, all exhibited this property. Since then, three (CA1, EC1, and CA2) of the cultures have become avirulent. Nevertheless, these organisms have been incorporated in order to compare virulent with avirulent forms. The cultures used were, with a few exceptions, identical with those studied by Elrod (1941) in a biochemical and serological survey of the soft-rot group. They were all actively motile.

EXPERIMENTATION

All of the biochemical and rotting tests were conducted in duplicate at both 20°C and 37°C to ascertain if *Erwinia* exhibited the same temperature differences noted by Stuart *et al.* (1940) in their study of aberrant coliforms.

a. Lactose fermentation

As is seen in table 1, all of the *Erwinia* cultures were able to ferment lactose at 20°C. The time necessary for the production of acid (Durham fermentation tubes with brom-cresol-purple as an indicator were employed) varied considerably. Several cultures, NZ, 4, 5, 11, EC1 and B81, formed acid in 24 hours. The slowest fermenting culture was ES which required seven days to produce a definite acid reaction. A small bubble of gas was produced by the tenth day. Gas was formed by seven of the strains but never in a quantity exceeding 20 per cent of the vial. This gas formation was slow and only an immeasurable amount was formed in 48 hours. Whereas all the cultures fermented the disaccharide at 20°C, five failed to do so at 37°C.

b. IMViC plus cellobiose

None of the cultures tested for the formation of indole with Kovac's reagent formed this substance in five days' time in tryptophane broth (table 1). This

result is in keeping with most of our information concerning indole production by soft-rot organisms. Exceptions can be found in the literature, most notably in Bergey *et al.* (1939) where *Erwinia solanisapra* and to some extent *E. carotovora* are said to form small amounts of indole. Stanley, however, with over 100 cultures found but two organisms which formed indole and one of these was a non-lactose-fermenter. Bonde (1939) was able to demonstrate indole production by several soft-rot strains by means of Kovac's test, although several other tests had proved negative.

TABLE 1

Lactose, IMViC and cellobiose reactions by Erwinia isolates at 20°C and 37°C

ORGANISM TESTED	LACTOSE		INDOLE		METHYL RED		VOGES-PROSKAUER		CITRATE		CELLOBIOSE		IMViC + CELLOBIOSE AT TEMPERATURE OF GREATEST ACTIVITY
	20°	37°	20°	37°	20°	37°	20°	37°	20°	37°	20°	37°	
CA1	AG	AG	−	−	±	+	+	+	+	+	AG	AG	−++++
NZ	A^b	A	−	−	+	+	−	−	+	−	A	A	−+−+A
496	A	−	−	−	+	−	−	−	−	−	A^b	A	−+−−+
494	A	−	−	−	+	−	−	−	+	−	A	−	−+−+A
WV3	A	A	−	−	+	+	−	−	+	+	A	A	−+−+A
EC1	AG	AG	−	−	−	−	+	+	+	+	A^b	+	−−+++
EC2	A^b	A	−	−	+	−	−	−	+	−	A	A	−+−++
EC4	A	A	−	−	−	−	−	±	+	−	A	A	−−++A
EC3	A	A	−	−	−	−	−	±	+	−	A	A	−−++A
B81	A	A	−	−	+	±	+	±	+	−	A	A	−+++A
5	A	A	−	−	+	−	−	−	+	−	A	A	−+−+A
11	A	A	−	−	+	+	−	−	+	+	A	A	−+−+A
EA	A	A	−	−	+	−	+	−	+	−	A	A	−+++A
ES	A^b	−	−	−	+	−	−	−	−	−	A	−	−+−−A
495	A	−	−	−	+	±	−	−	+	−	A^b	A	−+−++
EC	A	A	−	−	+	+	−	−	+	+	A	A	−+−+A
WV6	A	AG	−	−	−	−	+	+	+	+	AG	AG	−−+++
CA2	AG	−	−	−	+	±	+	+	+	+	AG	−	−++++
4	A	A	−	−	+	−	−	+	+	−	A	A	−+−+A

AG = acid and measurable gas. − = no acid or gas or no reaction.
A^b = acid and bubble of gas. ± = weak reaction.
A = acid only. + = strong reaction.

The medium used in both the methyl-red and Voges-Proskauer test was Difco's M.R.-V.P. medium. Methyl-red tests were made at 48 and 96 hours, while acetylmethylcarbinol was tested for each day for one week. In the latter, Barrit's α-naphthol test was used. The Voges-Proskauer test was fairly constant at the two temperatures employed, while a considerable difference was noted in the methyl-red reaction between 20°C and 37°C. A stronger acid reaction with ten of the cultures was produced at the lower temperature. In the final analysis, eleven cultures were MR+, VP−; four, MR−, VP+; and four, MR+, VP+ (table 1).

The methyl-red and Voges-Proskauer tests seldom have been used in regard to *Erwinia* isolates. Dowson (1939) lists *E. aroideae* as VP+, MR− and

E. carotovora, VP−, MR+. Whether more than one culture of each was used in drawing this conclusion is not known. Stanley found his cultures to be extremely variable in the methyl-red test when tested over a period of years. Many of his cultures were VP−, MR+, many VP+, MR−, and a larger number VP−, MR−, while only a few were VP+, MR+.

All of my cultures but two (ES and 496) were positive on Simmon's citrate agar at 20°C. At 37°C, however, only seven of the strains were able to utilize the citrate as the sole carbon source (table 1). The majority of Stanley's cultures were citrate-positive at 20°C.

TABLE 2

Gelatin liquefying, pectin fermentation and rotting tests of Erwinia and coliform cultures

ORGANISM TESTED	GELATIN	PECTIN	CARROT	TURNIP
CA1	+	+	−	−
NZ	+	+	+	+
496	+	−	+	+
494	+	+	+	+
WV3	−	+	+	+
EC1	+	−	−	−
EC2	+	+	+	+
EC3	+	+	+	+
EC4	+	+	+	+
B81	+	+	+	+
5	+	+	+	+
11	+	+	+	+
EA	+	+	+	+
ES	+	+	+	+
495	−	+	−	+
EC	+	−	+	+
WV6	−	−	−	+
CAZ	+	+	−	−
4	+	+	+	+
50 representative coliforms		22*	0*	0*

* Number of cultures reacting positively.

As in the case of lactose, all of the strains fermented cellobiose at 20°C. In some cases the reaction proceeded rapidly; in others, very slowly. Three isolates failed to ferment the sugar at 37°C (table 1).

c. Gelatin liquefaction

One of the differences between *Aerobacter aerogenes* and *A. cloacae* is the ability of the latter to liquefy the gelatin medium. This reaction, however, is not a constant characteristic, there being found numerous non-liquefying strains of *A. cloacae*. Gelatinolysis with this organism usually proceeds slowly, although there are exceptions.

In gelatin stab cultures, sixteen of the nineteen isolates were able to hydrolyze the medium (table 2). For the most part, this liquefaction was rapid,

being complete in 2–3 days at 20°C. There were a few cultures that required 10–14 days before hydrolysis was detectable. Frazier gelatin-agar plates also proved the remaining five strains to be negative. Bonde (1939) has indicated that all of his isolates liquefied gelatin, and, this is usually thought to be true of soft-rot organisms.

d. Pectin fermentation

Inasmuch as the reducing sugars associated with pectin are soluble in 80 per cent alcohol, several soakings and washings with this solvent eventually removed any of these contaminating substances. The usual procedure was to place a weighed sample of the granulated pectin in a flask to which considerable 80 per cent alcohol was added. The mixture was shaken thoroughly and then incubated 12 hours with occasional shaking. The alcohol was filtered off, fresh alcohol added and the flask again incubated 6–8 hours. The mixture was filtered and the pectin on the filter paper washed 6–7 times with 80 per cent alcohol and twice with 95 per cent. The alcohol-moist pectin was then placed in a sterile petri dish and heated to dryness at 37°C. A 5 ml. sample of the last 80 per cent alcohol filtrate was evaporated to dryness and a qualitative Benedict's test performed with the residuum; this invariably proved negative for reducing sugars. This procedure shortens the time of McFadden's method (1941) five or six days.

A basic synthetic medium was made up of 0.2 g. magnesium sulfate, 0.1 g. calcium chloride, 0.2 g. sodium chloride and 0.2 g. dipotassium phosphate per liter. This was sterilized by filtration through a Berkefeld N filter. The pectin was added so that the final concentration was approximately 0.5 per cent. Brom-cresol-purple was used as an indicator. On the addition of pectin, the medium became acid and it was necessary to adjust to neutral with sterile NaOH. After tubing aseptically, the tubes were incubated 48 hours at 37°C and 3–4 days at 20°C. The percentage of contaminated tubes was about 5 per cent; fairly effective sterilization of the pectin had taken place during the purifying process.

Four tubes of this medium were inoculated with each *Erwinia* and coliform culture, two incubated at 37°C and two at 20°C for one week. All but two (EC and 496) of the actively rotting organisms fermented pectin. On the other hand, there were a great number of *Aerobacter* and intermediate coliform cultures which fermented the carbohydrate. Only three strains of *Escherichia coli* were positive in the pectin medium; all of these were of fecal origin. Among the fermenting *Aerobacter* cultures a large number were derived from feces. It is apparent that there is no correlation between the ability to ferment pectin and the rotting of carrot or turnip (table 2).

e. Pathogenic action on carrot and turnip

As a means of testing the pathogenicity of the organisms, both carrots and turnips have been used. Jones' (1905) method was used to test the pathogenicity of the organisms against carrot and turnip. The vegetable tissue was sectioned aseptically and one piece (0.5 cm. x 0.5 cm. x 1.0 cm.) added to a tube

of nutrient broth and incubated several days to control for contamination. Tubes of both carrot and turnip were inoculated in duplicate; one set was incubated at 37°C and the other at 20°C. The progress of the rot was tested by probing with a stiff nichrome needle. Those showing active rot became soft and soon broke up. Unaffected pieces and controls in uninoculated broth were firm after weeks of incubation. This method proved far more satisfactory than inoculating sterile slices of carrot or turnip. This test is in reality a test of protopectinase production (Davison and Willaman, 1927).

The ability to macerate the vegetable tissue was possessed by sixteen of the *Erwinia* isolates (table 2). Five of the cultures (CA1, CA2, EC1, 495 and WV6) failed to act on the carrot, while three (CA1, EC1 and CA2) also had no effect on turnip. Inasmuch as all of the cultures were known at one time to be pathogenic for carrot and turnip, the ability to rot must have been lost in storage. The lower temperature was more effective, although some organisms were able to act on the middle lamella of the cells at 37°C.

Fifty coliform organisms including *Aerobacter aerogenes*, *A. cloacae*, *Escherichia freundii*, and *E. coli*, as well as intermediates, isolated from soil, grain, feces, etc., were tested similarly. In no case, however, was the slightest degree of maceration detectable (table 2). These tests were examined daily for three weeks and in each case the vegetable tissue remained as firm as the controls in uninoculated broth.

DISCUSSION

Had the *Erwinia* cultures used in this work been isolated in the course of a coliform investigation, there seems little doubt but that most would have been classified as aberrant or irregular coliforms. Without the aid of maceration experiments, there is no possible means by which they could be classified elsewhere. Classifying these organisms according to Stuart's aberrant coliform grouping, six are micro-aerogenic, while the others are anaerogenic. It is known, however, that, like coliforms, *Erwinia* cultures can shift from an anaerogenic state to an aerogenic one, or vice versa (Stanley 1939, Elrod 1941). It was originally contended by Harding and Morse (1909) that the anaerogenic soft-rot organisms were only strains of the aerogenic forms, and most certainly Dowson's (1941) recent separation of *Erwinia aroideae* and *E. carotovora* will break down due to this fact. Thus, the aerogenic and anaerogenic states in both the coliform group and *Erwinia* are not constant.

By means of the IMViC reactions plus cellobiose fermentation, *Erwinia* isolates can be placed in well-known coliform groups. Nine of the cultures were either group 8, $-+-++$, or group 9, $-+-+$A (Stuart's classification); four were in groups 2 and 3, $--+++$ and $--++$A; one was group 11, $-+--+$; one was group 12, $-+--$A; while four were grouped as irregulars, $-++++$ or $-+++$A. The majority of these fall into the group classified by Stuart as intermediates; none showing any apparent relationship to the *Escherichia coli* group. From this work it seems that the majority of the soft-rot organisms more nearly approached the characteristics of *E. freundii*, $-+-++$.

Gelatinolysis occurs more rapidly with *Erwinia* than with *Aerobacter cloacae*, at times the speed of reaction more nearly approaching the *Proteus* group. The fact that all of our *Erwinia* cultures are motile would likewise link the group with *A. cloacae*. It has also been shown by Elrod (1941) that a large majority of *Erwinia* strains ferment glycerol with the formation of acid only, seldom acid and gas. This also is characteristic of *A. cloacae*. The closer affinity of the soft-rot group would seem, therefore, to lie with *A. cloacae*, and not *A. aerogenes*. Depending on the criteria used, it would be possible to link *Erwinia* with either *Escherichia freundii* or *A. cloacae*.

The three pectic substances associated with plant tissues are protopectin, pectin and pectic acid. It is the protopectin which forms the cementing material of the middle lamella of plant cells. On hydrolysis protopectin yields an araban and pectin, and the binding property of the substance is lost. The separation of the cells from one another is usually spoken of as maceration. Macerating can be effected in many ways: long boiling (especially under pressure), treatment with 0.5 per cent ammonium oxalate at 70°–80°C, heating with dilute acids, electrodialysis and by the enzyme protopectinase. It is the latter which gives effectiveness to the soft-rot organisms and to certain fungi. Pectin fermentation, on the other hand, is undoubtedly due to several enzymes; pectinase, which hydrolyzes pectin and pectic acid to sugars and galacturonic acid, and the enzymes which would act on these sugars. It is apparent, therefore, that there is no reason to expect that the ability to ferment pectin is any indication of an invasive nature. From the experiments conducted here, it is obvious that coliform organisms do not possess the ability to liberate protopectinase and therefore cannot assume the role of attacking plants. This protopectinase activity would seem to be a criterion of sufficient importance to separate virulent *Erwinia* isolates from other lactose fermenters. It is difficult, however, to determine the taxonomic position of soft-rot organisms which have lost the ability to produce protopectinase. It seems, at present, that the ability to rot living plant tissue is a characteristic which warrants maintaining the soft-rot organisms in a position distinct from coliform bacteria, i.e., in the genus *Erwinia*. At the same time, however, one must recognize the very close relationship of the two groups. It is also true that such descriptions as "usually attack pectin" as given in Bergey's manual are entirely misleading.

CONCLUSIONS

The manner in which species of *Erwinia* ferment lactose would tend to classify them as aberrant coliforms. On the basis of their IMViC patterns, the majority would seem to approximate *Escherichia freundii*. On the other hand, according to their gelatin-liquefying ability, motility and production of acid in glycerol, the relationship seems to be closer to *Aerobacter cloacae*.

Sixteen of the *Erwinia* cultures possessed the ability to macerate vegetable tissue. This characteristic was not shared by any of the 50 coliform cultures. All but two of the *Erwinia* isolates fermented pectin in a synthetic medium, while 22 of the 50 coliform organisms, including three *Escherichia* of fecal origin, did so. There was no correlation between the ability to disorganize plant

tissue and pectin fermentation. On the basis of this macerating ability, it is contended that the placing of soft-rot organisms in a genus (*Erwinia*) separate from the coliforms is valid. At the same time the close relationship of the two groups is recognized.

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